WATER WATER WATER WATER WATER WATER WATER WATER WATER WATER

PROJECT COMPLETION
REPORT NO. 373X

The Ecologic Impact of the Interactions Among Microorganisms and Aquatic Contaminents In Lake Erie, Phase III

PARTS 5, 6 AND 7

By
Robert M. Pfister
Patrick R. Dugan
James I. Frea
Chester I. Randles

United States Department
of the Interior

CONTRACT NO.
B-025-OHIO
MG 14-31-0001-3320

The Ecologic Impact of the Interactions Among Microorganisms and Aquatic Contaminants in Lake Erie Phase III
Parts 5, 6, and 7

by

Robert M. Pfister
Professor

Patrick R. Dugan
Professor

James I. Frea
Associate Professor

Chester I. Randles
Professor

Department of Microbiology

This study was supported in part by the Office of Water Resources Research, U. S. Department of the Interior, under Project B-025-OHIO.

Students involved in this work who obtained the Ph. D. degree:

Patricia A. McCabe	Part 5
Jayne Frances Carney Martin	Part 6
William Claus Dierksheide	Part 7

TABLE OF CONTENTS

Part 7 Page

Part 5

Interactions among microbial cells, enzymes, substrates and clay minerals.

Part 5

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

In aquatic environments, large quantities of organic materials, some very complex are recycled by the enzymatic activities of micro-organisms. Microbial extracellular enzymes responsible for decomposing organic substances have been extensively investigated under laboratory conditions, but one natural factor rarely considered in enzymological studies is the ubiquity of mineral particulates in aquatic ecosystems. That clay minerals do influence microbial metabolism and enzyme activity in soils has been well documented, but little is known about the overall effect that such particulates have on the decomposition of refractory organic compounds in lakes.

Collagen, chitin and celloulose are synthesized abundantly by aquatic ogranisms. For producers, the durability of these compounds is advantageous; for those organisms, man included, which would recycle the components of such compounds, durability presents an obstacle. The endurance of an organic compound is a function of the abundance of hydrolyzing agents and the accessibility of the compound of those agents. Man considers collagen, chitin and cellulose refractory only because relatively few organisms produce the requisite hydrolytic enzymes necessary to decompose them.

As a group, the actionmycetes are considered able to degrade these complex organic materials. No data are available in lakes, but their high numbers relative to the total bacterial count, the broad range of their enzymatic competence, the rapidity with which some strains convert complex organic materials into cell mass under laboratory conditions, and the absence of large deposits of arthropod shells, animal hides, dead leaves and similar detritus in lake bottom sediments, suggest that actionomycetes may contribute significantly to degrading these materials. If actinomycetes are active in recycling refractory compounds in lakes, they may be valuable for recycling the same materials as they become the waste products of man's industry.

In composting, man has long exploited the cellulolytic capability of soil actinomycetes to convert unwanted plant products into usable soil amendment. He may well be able to harness the protelytic and chitinolytic facility of aquatic actinomycetes to convert hide, hair, feathers, fish scales and arthropod shells into usable carbohydrates and amino acids.

Since mineral particulates significantly influence microbial metabolism and enzyme performance, we undertook a study of some aspects of this influence with a view to its pertinence to problems of waste disposal and lake eutrophication. We have demonstrated, usually qualitatively, occasionally quantitatively, five interactions which affect the enzyme activity of microorganisms of the family Streptomycetaceae and committantly affect the degradation of the organic materials which they utilize. These five interactions are:

1. Adherence of mineral particularly of cells.
2. Adherence of microbial cells to three particulate substrates: collagen, chitin, and cellulose.
3. Adherence of kaolinite to collagen.
4. Adsorption of extracellular enzymes to mineral particulates.
5. The activity of absorbed enzyme when the adsorbent mineral adheres to a particulate substrate, collagen.

LITERATURE REVIEW

Decades ago microbiologists were aware that addition of particulates to liquid cultures of bacteria could stimulate growth (Heukelekian and Heller, 1940). Conn and Conn (1940) suggested that increased surface for growth provided by mineral colloids, or their adsorption of inhibitory waste products, rather than just a contribution of ions to their medium, might explain the increase in microbial growth. Bigger and Nelson (1941) inferred that talc, serving as a surface for adsorption of CO_2 and ammonia dissolved in the distilled water menstruum acted as a surface catalyst, enabling coliform bacteria to subsist on laboratory air as a source of carbon and nitrogen. ZoBell (1943) concluded that solid surfaces concentrated adsorbed nutrients from very dilute solutions, provided a resting place for sessile bacterial, and adsorbed extracellular enzynes and their hydrolysates, bringing organism, enzymes and substrates into close contact. He inferred that surface absorption reduced the probability that exoenzymes would diffuse away from the producer or nutrients from the prospective consumer during hydrolysis.

ZoBell's conclusions seem reasonable in view of the available data concerning adsorption of organic materials. Because the conditions under which clay minerals interact with simple and complex organic molecules are of such importance to agriculture and waste water treatment, they have been studied extensively.

Silicate clays, the characteristic soil colloids of temperate regions, consist mainly of montmorillonites, e.g. bentonite; the hydrous micas, of which illite is an example; and the kaolinites. These colloidal - hence biologically important - portions of the soil and the silt loads of rivers, lakes and oceans, represent three stages in the weathering of feldspars and micas. All are ionic lattices with loosely associated, hydrated, exchangeable cations (Buckman and Brady, 1960).

Montmorillonite consists of an alumina layer tightly bound between 2 hydrated silica layers by mutually shared oxygen atoms. These 3-layered units are loodely associated with each other by O-O bonds which easily allow expansion of the lattice. Montmorillonite crystals fracture easily into individual particles (micelles) having diameters ranging from 0.02 μm and thicknesses ranging from 0.002 μm.

Illite, also a 3-layered silicate clay mineral, has about 15% of the silicon ions of the middle layer replaced by aluminum. Potassium ions are generally associated with the aluminum ions, balancing the charge deficit created by the ion substitution. This type of lattice is less expansive than that of montmorillonite. The individual particles are generally larger, with 0.1 μm to 0.2 μm diameters.

Kaolinite, formed from montmorillonite, or directly from parent rock by weathering which removes alkali and alkaline-earth metals, is a 2-layered structure with 1 alumina and 1 hydrated silica layer in a non-expanding lattice. The dimensions of flake surfaces range from 0.3 μm to 4.0 μm and thicknesses from 0.05 μm to 2.0 μm.

On paper, the structures described for these 3 clay minerals are ionically balanced. But experimental evidence points overwhelmingly to the fact that the mineral particles

carry not only net charge, but point charges, both positive and negative. Three mechanisms have been advanced as possible sources of these charges: isomorphous replacement, ionization of water of hydration and lattice defects. Isomorphous replacement of Si by Al, or of Al by Mg would produce a net negative charge on the crystal faces and could account for the high surface charge and ion exchange capacity of montmorillonite (ca. 100 meq/100 g) and illite (10 - 4 meq/100 g) relative to kaolinite (2) 4 meq/100 g) (Wayman, 1967). Ionization of water molecules bonded to aluminum along the edges of micelles would create sites of positive charge in acid environment, negative charge in alkaline solutions. This could explain why the cation exchange capacity of clay minerals increases with increase in pH. Under acid conditions, water of the alumina layer could adsorb H^+ or other cations and neutralize the site, creating a positive surface charge. Lattice defects, due to leaching of aluminum hydroxide in acid solutions, would result in a net negative charge. Cashen (1966) remarked that the argument is not over the existence of positive edge charges on clays, rather it is over their source and performance.

Whatever their origin, unbalanced surface charges can be satisfied by adsorption of other charged particles. Clays are good adsorbents because of their large surface area (hence high total surface charge) per unit weight. The expanding lattice of montmorillonite, by presenting interlamellar as well as micellar surfaces to solutions, makes this type of clay a much more efficient absorbent than non-expanding kaolinite (Tailbudeen, 1951). Adsprption of organic molecules to clay minerals at pH's thought to render both species anionic may be due to cation bridging.

Some authors have adduced data indicating that forces other than ionic bonding are responsible for sorption of organic materials to clays (Mattson, 1932; Lynch, Wright, and Cotnoir, 1956; Bader, Hood, and Smith, 1960). But Evans and Russell (1959) found that adsorption of fulvic and humic acids to kaolinite and bentonite was independent of temperature, and related to the cation of homoinonic clays. These data indicated that Coulombic forces rather than hydrogen bonding or Van Der Waal;s forces were involved.

Ensminger and Gieseking (1939) suggested that at pH's below their ioselectric points (pI's), proteins adsorb to bentonite as cations. In 1942, these same authors related the ability of kaolinite and bentonite to absorb protein with the base exchange capacities of the two minerals. Hendricks (1941) stated that large organic cations were held to montmorillonite not only by Coulombic forces between ions, but also by Van der Waal's attractions between surfaces.

McLaren (1954a) studies the relationship between the pI's of 9 proteins and their adsorption to kaolinite as a function of pH. Since kaolinite acts as a weak acid above ca. pH 4, he used proteins with pI's above and below pH 4. For 7 of the proteins, maximum adsorption occurred at pH's 1-3 units lower than the pI. He observed that decrease in binding ofproteins to kaolinite would be expected above the pI if net surface charges were solely responsible for binding. But even above the pI, some surface regions of the protein would bear positive charges and unless the molecule as a whole were highly negative, some binding of kaolinite would occur. His data bear this out; although binding dropped off below or at the pI, it did not fall abruptly to zero. In this, and in a later paper (1957), McLaren pointed out that adsorption on a solid surface alters the apparent pI of protein. The pH for optimal activity of chymotrypsin on kaolinite was 2 units higher than the optimal

pH in solution because the surface of the clay colloid was more acid than the solution proper.

Further elucidation of the nature of the clay-protein relationship at various pH's was reported by McLaren, Peterson, and Barshad (1958). In this paper, Peterson's interpretation of curves plotted for adsorption of lysozyme of kaolinite and bentonite in indicated maximum adsorption of lysozyme on both minerals occurred at pH's above the pI of the protein. As pH increases above 2, the net negative charge on clays increases and the net positive charge on protein decreases, becomes zero, then reverses to a negative charge above the pI. Thus the increasing tendency for a negative clay to adsorb a positive protein is counteracted by the protein's tendency to become less positive, and eventually negative The maximum adsorption of protein to clay near the protein's pI may be accentuated by the lessening of self-repulsion by the protein as its net charge approaches zero. But the adsorption maximum would be shifted away from the pI by the increased hydrogen ion activity on the clay mineral surface.

Investigations by soil scientists of clay mineral - organic associations were prompted by their need to understand the nature and extent of the resistance to microbial degradation afforded organic matter by soil particles. Ensminger and Gieseking (1942) found that kaolinite had no significant affect on hydrolysis of albumin or hemoglobin, but bentonite interfered with hydrolysis of these proteins by both pepsin in acid solution and pancreation in alkaline solution. They assumed that either the protein was oriented in such a way that enzyme could not attack it, or the enzyme itself had been adsorbed and denatured. Pinck and Allison (1951) reported that when gelatin was reacted with montmorillonite, the protein adhering to the outside of the micelles was digested. Gelatin occupying interlamellar spaces was not. Esterman and McLaren (1959) observed that kaolinite acted as a concentrating surface for adsorbed organic substrates and microbial exoenzymes, thereby accelerating enzymatic breakdown of substrate. Esterman, Peterson and McLaren (1959) contrasted the rate of hydrolysis of protein freshly adsorbed to montmorillonite from solution with the rate for dried and rewetted protein-clay complexes. The former hydrolyzed at rates nearly equal to those for non-adsorbed protein in solution, but the dried, rewetted complex was somewhat resistant to attack. It should be noted here that Ensminger and Gieseking's data were obtained with dried protein-montmorillonite complexes. Barbaro and Hunter (1965), studying the effect of kaolinite and bentonite on the biodegradibility of surfactants, found that both clays inhibited degradation, bentonite much more so than kaolinite. They concluded that the absorption of surfactant to clay minerals rendered inaccessible sites of enzymatic hydrolysis. Ambroz (1966) reported that in complexes of bentonite containing 15% protein, microbial degradation of the protein was retarded. At higher percentages of protein, protolysis was stimulated. Weber and Coble (1958) measured microbial decomposition of the pesticide diquat adsorbed to kaolinite and montmorillonite. Kaolinite-adsorbed diquat was oxidized at CO_2, but montmorillonite-adsorbed pesticide resisted oxidation to an extent comparable to the calculated degree to interlamellar penetration.

These data clearly indicate that protein adsorbed to clay minerals is subject to enzymatic attack if the appropriate enzymes can contact active sites on the adsorbed substrate. The next obvious question is whether adsorption of proteins which themselves have enzymatic activity, interferes with that activity. McLaren (1954b) found that adsorption of enzyme led to reduction or loss of activity of lysozyme, pepsin, trypsin and chymotrypsin.

Some activity of lysozyme and trypson could be recovered on elution of the enzyme with ethylamine. Fraser (1957) concluded that dense packing of enzyme on an imm bile surface led to less denaturation than would occur at a liquid:air interface. As before, the nature of the clay mineral, whether kaolinite or montmorillonite, was very important. Talibudeen (1955) found from X-ray diffraction data that trypsin adsorbed to montmorillonite had unfolded and lost activity.

The previous studies were made with a view to explaining soil-organic matter interactions. But in another context, clay minerals were being used to purify enzyme preparations of impurities (Dixon, 1926; Adams and Hudson, 1943). "Success" varied with enzymes adsorbents and authors' points of view. One writer reported "elution of enzyme in active form" but another author, citing the same data, says "addition of an adsorbent to the system markedly reduced enzyme activity."

Since the publication of ZoBell's hypotheses about the effects of mineral surfaces on microbial metabolism (1943), several microbiologists have reported either enhancement or inhibition or microbial growth by various minerals, chiefly kaolinite and montmorillonite. Meadows and Anderson (1966) postulated that sand grain surfaces provided distinct and characteristic microhabitats for clones of marine microorganisms. Lee and Hoadley (1957) stated "In open water, bacteria may exist freely as single cells or associated with particulate matter... The surfaces of particulate matter, where organic substances are adsorbed and excreted products of other community members are available afford the associated bacteria and opportunity to function in an enriched situation relative to the surrounding open water. Colonization of surfaces may afford an opportunity to create special localized conditions, permitting... metabolism of certain organic materials by altering the pH, redox potential or by preventing dispersal of extracellular enzymes."

Cralley (1968) found that both kaolinite and bentonite simulated growth of actinomycetes in liquid cultures containing either soluble or particulate carbon sources; kaolinite produced a greater effect. Novakova (1968) reported that Na^{+} and Ca^{++} forms of bentonite shortened the lag phase of Escherichia coli cultures by about 75%, but lowered total cell yield. Kaolinite lengthened the lag phase and also diminished total cell yield. With both minerals, an increase in concentration produced an increase in effects. Pfister, Dugan, and Frea (1968), using particulates of various sizes removed from Lake Erie water by differential centrifugation, found that stimulation or inhibition of microbial growth varied with particle size and with the type of microorganism. Button (1969) added bentonite to single-phase continuous cultures of yeasts and E. coli to measure adsorption of the limiting nutrients, glucose and thiamine. He concluded that no significant adsorption occurred, attributing a temporary reduction of cells in one culture in unbuffed medium to perturbation caused by an increase in free heavy metal concentration.

Other authors have offered other explanations for effects produced by minerals in microbial cultures. Stotzky, in a series of papers (Stotzky and Rem, 1966; Stotzky, 1966a; Stotzcy, 1966b; Stotzky and Rem, 1967) reported that montmorillonite in soils favored growth of bacteria over fungi by exchanging clay-associated cations for the hydrogen ions produced by microbial metabolism; thus maintaining soil pH at a level more favorable for bacteria.

There is a real dearth of data concerning the effects of clay minerals on the growth

of aquatic microorganisms (Wayman, 1967). An example of the kind of oversight prevalent in the literature of Stotzky's statement: "Presence of mineral particulates is a feature which distinguished soil from other habitats." (Stotzky and Rem, 1966).

Another aspect of protein-to-mineral adsorption which has begun to receive attention is flocculation. Bader and Jeffrey (1958) studied the efficiency of kaolinite, illite and montmorillonite in removing radioactive organic matter from sea water. Much literature describes the behavior of polyelectrolyte polymers as mineral flocculants or dispersants (Ruehrwein and Ward, 1952; Heller and Pugh, 1960; Ulinska and Huppenthal, 1966; Black, Birkner, and Morgan, 1966; Mukherjee and Chakravarti, 1968; Kitchener, 1969). Busch and Stumm (1968) considered bacterial cell-surface material as a natural polyvalent polymer effecting flocculation. Ulinska and Huppenthal (1966) and Narkis, Rebhun, and Sperber (1969) warned that "model systems" involving purified minerals react very differently from natural systems in which clays are covered with soil salts and polymers. Although clay minerals are readily reconized as adsorbents in natural water purification processes, their possible use in conjunction with activiated sludge seems to have been largely ignored.

The actinomycetes have been extensively studies for their antibiotic producing qualities. Less attention has been paid to their ability to degrade many resistant organic materials such as collagen, chitin and cellulose. Erikson (1941) studied the cellulolytic and chitinolytic capacity of many strains of Micromonospora. Reese, Siu, and Levinson (1950; Reese, Smakula, and Perlin (1959); Hagen, Reese, and Stamm (1966); Waksman (1967); Enger and Sleeper (1965); Mandel (1969); and Jagow (1969) have reported on the cellulases of Streptomyces. Reynolds (1954); Berger and Reynolds (1958); Skujins, Potgieter, and Alexander (1965) and Jagnow (1969) have studies the chitinases of Streptomyces. Hagihara (1960) in a review of proteases, mentions the work of Nomoto and Narahashi (1959) who found that a prptease of Streptomyces griseus was as active as the B. subtilis enzynes generally used commercially. Cralley (1968) studied the effect the clay minerals kaolinite and bentonite had on the growth of Streptomyces and Micromonospora cultured in defined media with cellulose as a carbon source or chitin as a carbon and nitrogen source.

McCabe and Frea (1971) reported that addition of kaolinite to a cell-free solution of extracellular enzymes produced a streptomycrte, concentrated the enzyme from dilute solutions and brought it into intimate contact with particulate substrate. These data seem to confirm the hypothesis that mineral particulates influence the metabolism of microorganisms by serving as adsorbent surfaces for nutrients and enzymes.

MATERIALS AND METHODS

Organisms

Cultures of Streptomyces fradiae 5063 (Waksman), and Micromonospora chalcea ATCC # 12452, from stocks maintained by the Ohio State University Culture Collection, were used for preliminary studies. Three strains of Streptomyces and 4 of Micromonospora, isolated from Lake Erie, were screened for substrate utilization and ability to grow well in chemically defined broth medium. Of these, one streptomycete, here designated S-1, was used for all

studies of interactions among cells, extracellular enzymes, substrates and clay minerals.

Media

Yeast Extract Agar (YME) (Pridham et al., 1956/1957)

Slants of YME at pH 7.6 were used for maintaining all stock cultures. YME plates were used to check purity of broth cultures and to culture actinomycetes isolated on other media, to determine color and appearance of substrate and aerial mycelia.

Tryptone, Yeast Extract Broth (TYE) (Pridham and Gottlieb, 1948)

TYE, at Ph 7.6 was used for routine culture of S. fradiae and M. chalcea, to obtain cells for subsequent inoculation into chemically defined media. TYE cultures of the 7 organisms isolated from Lake Erie were both lyopholized and frozen in liquid nitrogen to preserve the original strains.

Mineral Salts Medium (MSM)

The carbon utilization medium of Pridham and Gottlieb (1948), was modified to eliminate precipitates which interfered with dry weight determination and optical density estimation to cell mass. MSM contained per liter:

Trizma Tris(hydroxymethyl) aminomethane and hydrochloride (Sigma)	0.1210 g
Maleic anhydride	0.0980 g
K_2HPO_4	1.1300 g
$MgCl_2$	0.4130 g
Trace Salts:	
$CuSO_4 \cdot 5H_2O$	0.0064 g
$FeSO_4 \cdot 7H_2O$	0.0079 g
$MnCl_2 \cdot 4H_2O$	0.0015 g
$ZnSO_4 \cdot 7H_2O$	0.0015 g

The pH was adjusted with NaOH to 8.2–8.3. The final pH after autoclaving was about 7.6. MSM was used with substrates containing both carbon and nitrogen to test organisms' ability to utilize the substrate as source of those elements.

Mineral Salts Medium with $(NH_4)_2SO_4$ (MSM-SO_4)

Ammonium sulfate, 1.64 g/liter, was added to MSM for use with substrates containing carbon, but no nitrogen, to test organisms' ability to utilize that substrate as a carbon source.

Mineral Salts Medium with 0.01M $CaCl_2$ (MSM-$CaCl_2$)

(MSM-$CaCl_2$) was used with native collagen to test for specific collagenase activity. MSM was used also with autoclaved collagen to determine whether Ca^{++} influenced

utilization of denatured collagen by Streptomyces sp. S1.

Glucose, Asparagine, Ammonium Acetate Medium (GM)

MSM containing per liter, glucose, 4.0 g; and L-asparagine· H_2O, 0.5 g, was autoclaved and cooled. Filter sterilized CH_2COONH_4 in distilled water was addes aseptically to give a concentration of 4.93 g/liter. This medium was used for routine culture of S-1, and for culture of washed S. fradine or M. chalcea cells harvested from TYE cultures. In some experiments, Trizma concentration was doubled (0.2420 g/liter) to increase buffering capacity. Maleic acid, wihch may have inhibited growth, was omitted from GM or MSM. These media are designated GM-2TB, MSM-2TB or MSM-SO_4-2TB.

GM Agar

Agar, 20 g/liter, was added to the autoclavable components of GM and sterilized. Filter sterilized ammonium acetate solution was added aseptically to the medium cooled to 47 C. Plates of this agar were used to test organisms' ability to grow on chemically defined solid medium.

Cellulose Agar

Coil cotton (Kendall Co., Walpole, Mass.) was hydrolyzed by Hungate's (38) method, but not milled. The pH was adjusted to 7.0 with NaOH and the slurry washed until the $AgNO_3$ test for chloride was negative. Cellulose at 1 mg/ml was added to MSM-SO_4-2TB containing 2% agar, pH 8.2.

Starch, Casein Agar and Actinomycete Isolation Agar

Starch, casein agar (JEN) (Jensen, 1930) and Actinomycete isolation agar (AIA) (Difco Supplementary Literature, September, 1966) were used to isolate Streptomyces and Micromonospora from Lake Erie water and bottom mud. To both media, Rose Bengal (Matheson, Coleman, and Bell, C. I. 45440) was added at concentrations of 1:20,000; 1:30, 000; or 1:40,000 to curtail bactarial and fungal growth (Smith and Dawson, 1944) without completely inhibiting acinomycete growth.

Substrates Tested

Collagen (Sigma); chitin, blood fibrin and keratin (Nutritional Biochemicals); cellulose (Whatman, CF11, W/R Balston, Ltd., England) and hydrolyzed cotton (Kendall Co., Walpole, Mass.); cellobiose (Pfansteil); casein (Bacto-Tryptone, Difco); Azocasein (Calbiochem) were used in substrate utilization tests.

Mineral Particulates

Kaolinite (Peerless Coating Clay) and illite (Fithian No. 35) supplied by Dr. Robert H. Miller, Department of Agronomy, The Ohio State University; and bentonite (American Colloid Co., Skokie, Ill.) were used, as supplied.

Isolation of Organisms

A sample of bottom mud and water was collected at a depth of 15 M from the Western Basin of Lake Erie. Four 10 ml portions of this sample were pipetted into sterile test tubes. The tubes were incubated in a 70 C waterbath with agitation, one tube per time period, for 0, 10, 20 or 30 min. The samples were used to streak plates of GM agar, Jensen's agar, or AIA, all containing various concentrations of Rose Bengal. The plates were incubated at 27 C. As colonies, apparently of Streptomyces or Micromonospora, were detected by stereoscopic microscopy at 250X, the colonies were picked off and each aseptically dispersed in a drop of distilled water. Some of the material was examined microscopically, with phase contrast, or stained with crystal violet, to determine cell morphology. If the cells appeared to be mycelial, the rest of the colony was used to streak fresh plates of the medium on which the colony had developed. When pure cultures were obtained on solid medium, the organism was cultured on YME, Jensen's agar, GM agar and AIA, all without Rose Bengal, to determine pigmentation. Appearance of substrate mycelium; of aerial mycelium, if any; spore morphology; pigmentation of the mycelia and medium as the organism developed on various media, and the ability to utilize the substrates tested, were used as criteria to distinguish strains. No further attempt was made to identify the strains isolated. Their behavior under my culture conditions was recorded, and they were assigned arbitrary OSU numbers. A second sample of Lake Erie water, collected from the same location, but at the surface on a calm day, was used for further isolation of actinomycetes. This sample was not heated prior to use; otherwise the same procedure was used.

Preparation of Standard Inocula

Cells cultured in TYE were centrifuged and washed twice in Tris-maleate buffer (0.01 M, pH 7.6) (26). Cells cultured in GM were washed once. Washed cells were resuspended in about 10 ml of buffer and homogenized with a TRI-R Stir-R (TRI-R Instruments, Jamaica, N.Y.) until the suspension contained no macroscopically visible clusters of cells. The cell suspension was diluted with buffer until its absorbance at 660 nm was 0.3. The diluted suspension was agitated continuously as samples were pipetted into flasks of test media. Dry weight determinations showed that separate filling of the pipette for each inoculation was necessary. If 1 ml or 2 ml inocula were dispensed sequentially from a 5 ml or 10 ml pipette, the inocula contained sequentially increasing amounts of cell material. Dry weight determinations of triplicate samples of inoculum were made in experiments in which the absolute increase of cell mass during incubation was to be determined.

Tests of Substrate Utilization

Weighed amounts of substrates were added to MSM or MSM-SO_4, at concentrations calculated to provide the same carbon and nitrogen ratio as that of one type of control: MSM-SO_4 with 4.0 g/liter of glucose. Another control, MSM with no added carbon or nitrogen source, was included in these experiments. When particulate substrates were used, increase in cell mass was judged visually, sometimes recorded photographically. Microscopic examination of the cell morphology was sufficient to determine whether normal growth or autolysis were occurring. When soluble substrates were tested, cultures were centrifuged and dry weights of the pellets determined.

Spectrophotometry

Optical densities were determined with a Spectronic-20 (Bausch and Lomb) spectrophotometer. Matched Pyrex tubes were used for reading absorbance at wavelengths below 625 nm. There was no significant difference between disposable soft glass 13 x 100 mm culture tubes at wavelengths above 625 nm. These were used in sedimentation tests where suspensions could not be transferred to a cuvette during the settling period.

Estimation of Protein

The total protein content of Fluids was estimated by the method of Lowry, et al., (1951) as described in Manual of Microbiological Methods (46). Absorbance of 3 replicates of each sample were read at 660 nm. Triplicate samples of freshly prepared solutions of Bovine serum albumin (BSA) in 0.01 M phosphate buffer at pH 7.0, at concentrations of 0, 30, 60, 90, 120 and 150 μg of protein per 0.5 ml sample, were included in each determination. Protein concentrations of experimental solutions were calculated from standard curves computed from BSA data.

Assay of Enzyme Activity

Rate of dye release from substrates conjugated with azo chromophores was used to assay for enzyme activity (Oakley, Warrack, and Van Heyningen, 1946; Nelson, Ciacco, and Hess, 1961; Rinderknecht, et al., 1968). For substrates suspended in buffer, samples were centrifuged to remove suspended particles, and absorbance by dye in the supernatant fluid was measured spectrophotometrically at an appropriate wavelength. Dye concentration was calculated from a standard curve of known amounts of hydrolyzed substrate. Dye release from substrate particles included in agar pour plates was estimated visually and recorded photographically. Sufficient quantity of dyed substrate was used in each test to insure that at least 100 macroscopically visible particles were included in each plate. Decolorization of 90% of the substrate particles in a plate was used as an endpoint, since the rate of dye release from individual particles varied with the surface-to-volume ratio of the particles.

Density of Gradient Centrifugation

Sucrose

Discontinuous gradients of water and 65% (w/v) sucrose in water, were prepared in 12 ml conical glass centrifuge tubes. These were centrifuged at 2,000 x g for 15 min. Material was removed from the interface and from the bottom of the gradient with a Pasteur pipette.

Renografin

Renografin-76 (Squibb). To form each gradient, 1 ml of Renografin-76 (N-N'-diacetyl-3, 5-diamino-2, 4, 6-triiodobenzoate; density 1.45-1.46) was pipetted into a 20 ml

cellulose nitrate tube. Fourteen ml of a linear Renografin and water mixture was added from a linear gradient maker, and 1 ml of the sample was layered over the gradient. Tubes were centrifuged at 13,300 x g for 1 hour. Temperature was maintained at 2-4 C throughout formation and centrifugation of the gradient. Gradients were photographed as soon as possible after removal from the centrifuge, and were kept in an ice bath until fractionated. Fractions of 1 ml or 0.4 ml were collected upward by using Renografin as a propellant. The linearity of gradients was checked by measuring the refractive index of the Renografin solution from each fraction of a gradient, and comparing the values obtained with those of a standard curve based on the refractive indices of known concentrations of Renografin. Densities of individual fractions were estimated from the standard curve.

Estimation of Kaolinite-Cell Aggregation by Determination of Sedimentation Velocity

S-1 cells were homogenized or agitated in a Waring Blendor until mycelial clumps were dispersed. Samples of cell suspension were added to 13 x 100 mm culture tubes containing appropriate concentrations of kaolinite and of buffer adjusted to give a particular ionic strength and pH, at a final dilution of 6 ml. Triplicate samples of each level of the variables were used. The tubes were vortexed vigorously before a zero time absorbance was measured at 660 nm. Absorbance was measured at suitable time intervals, with care taken not to shake or jar the tubes. Changes in absorbance with time were plotted graphically and analyzed statistically.

Statistical Analyses

Programs written for the Smith-Corona Marchant Cogito Model 1016PR calculator were used to compute mean, standard deviation and standard error of mean; slope and intercept of linear portions of standard curves; and analysis of variance for one-way classification. Programs written for the Ohio State University CPS-360 computer were used for single variable, linear regression analyses, one-way analyses of variance and two-way analyses of variance.

RESULTS

Preliminary Studies of Streptomyces Fradiae and Micromonospora Chalcea

Laboratory strains of S. fradiae and M. chalcea were used to make preliminary tests of (1) substrate utilization; (2) adherence of mineral particulates to mycelium; (3) adherence of proteins in general, and enzymes in particular to mineral particulates, and (4) degradation of dye-conjugated collagen substrates by cell-free enzyme preparations. The preliminary tests indicated that (1) actinomycetes were suitable organisms for studying enzymatic degradation of complex organic materials; (2) mineral particulates adhered to mycelia and protein substrates; (3) active enzyme was absorbed to kaolinite and bentonite, and (4) the collagen preparations, Azocoll and HPA, used in conjunction with mineral particulates, permitted qualitatively sensitive assays of enzymatic activity.

Substrate Utilization by Streptomyces Fradiae and Micromonospora Chalcea

Standard inocula of S. fradiae or M. chalcea were added to 250 ml shaker flasks containing 50 ml of MSM with 1 g/liter of total substrate supplied in the following forms: GM; MSM + CH_3COONH_4 + cellulose; collagen; chitin and casein.

Growth was judged by the macroscopic appearance of the cell mass and by microscopic examination of the mycelia. Both organisms utilized all 4 complex substrates. M. chalcea's growth was poor in the cellulose medium. Both organisms grew much more extensively in complex media than in GM, a chemically defined, adequate medium. Both organisms cultured especially well in medium containing chitin, a polymer of N-acetylglucosamine. Growth was less rapid in media containing collagen or casein. The reason for this was not obvious, unless the chitin, a rather crude preparation, contained growth factors not present in the other media. Table 1 summarizes the results. Growth was rated relative to the cell mass produced by the organism cultured in GM, which was arbitrarily set at 1+ . The observations concerning pigment were included for those media in which suspended substrate could have been mistaken for cells were it not for the pigmentation; bright orange for the M. chalcea, greenish yellow for S. fradiae.

Adherence of Kaolinite to Mycelia of S. Fradiae and M. Chalcea

We wished to test whether adherence of kaolinite to the mycelia of S. fradiae and M. chalcea were stable enough, or too stable, to be used in conjunction with density gradient centrifugation to effect the following separations: (1) the organisms from each other in mixed culture; (2) organisms from particulate substrates, and (3) mineral adsorbed enzyme from cell surfaces.

Kaolinite was added to cultures of each organism in MSM + gelatin (1 g/liter); MSM + blood fibrin (1 g/liter), and TYE. Particulate materials in each culture were sedimented and a sample layered over a discontinuous water: 65% (w/v)sucrose gradient. Kaolinite adhered to some extent to all organic materials, but especially to M. chalcea mycelium and to blood fibrin. Table 2 describes the materials found at the water: sucrose interface and in the pellet of each gradient. Little kaolinite was found in the interface layer but much kaolinite and organic matter was aggregated in pellets. The density of a given suspended particulate seemed to depend more on the organic material-to-kaolinite ratio than on the nature of the organic material. A more discriminating gradient would be required to separate these organic materials. Kaolinite was not removed from particulate substrate or from cells by this type of centrifugation, which would be inappropriate for removing enzyme from cell surfaces by kaolinite adsorption.

Adsorption of Protein in General and Protease in Particular to Kaolinite and Bentonite

The first approach to this was to take samples of S. fradiae or M. chalcea culture medium, filtered to remove all cells and spores, and add kaolinite or bentonite to one sample,

TABLE 1

Substrate utilization by Streptomyces fradiae and Micromonospora chalcea.

Organism	Substrate									
	MSM[a] + glucose + asparagine + CH_3COONH_4		MSM + CH_3COONH_4 + cellulose		MSM + collagen[b]		MSM + chitin		MSM + casein	
	growth[c]	pigm.[d]	growth	pigm.	growth	pigm.	growth	pigm.	growth	pigm.
S. fradiae	1+	0	2+	1+	2+	0	3+	1+	2+	2+
M. chalcea	1+	0	±	1+	2+	0	3+	2+	2+	2+

[a]Minimal salts medium.

[b]Collagen was autoclaved; therefore denatured.

[c]Growth was judged relative to the increase in cell mass of the organism cultured in MSM + glucose asparagine + CH_3COONH_4, rated as +1.

[d]The observations concerning pigmentation were included for media in which suspended substrate could have been mistaken for cells were it not for the pigmentation; greenish yellow for the S. fradiae, bright orange for the M. chalcea.

TABLE 2

Distribution of Streptomyces fradiae and Micromonospora chalcea, blood fibrin and kaolinite in discontinuous density gradients.[a]

ORGANISM	MEDIUM	MATERIAL AT INTERFACE	MATERIAL IN PELLET
S. fradiae	TYE[b]	cells with some adherent kaolinite	cells much more dense, with large masses of adherent kaolinite
S. fradiae	MSM + gelatin	large dense colonies, not much associated kaolinite except small crystals adhering to the periphery of mycelial masses	large cell masses with large kaolinite crystals adhering to them; much dispersed or slightly aggregated kaolinite
S. fradiae	MSM + blood fibrin	2 layers: upper, of cells; lower of blood fibrin, both associated with kaolinite; very little free kaolinite	2 layers: upper, cells and mats of blood fibrin, but not much kaolinite associated with cells; lower, large flakes of fibrin and clusters of cells associated with mineral.
M. chalcea	TYE	no interface layer; 6 clumps of cells hung in upper portion of sucrose layer; some kaolinite associated with these.	2 layers: upper, mycelium heavily loaded with kaolinite but much free kaolinite also; lower, cell masses even more heavily loaded with kaolinite, large crystals of free mineral
M. chalcea	MSM + gelatin	dispersed cell masses heavily loaded with small kaolinite crystals	cells heavily loaded with kaolinite; little free mineral
M. chalcea	MSM + blood fibrin	continuous dense mesh of blood fibrin and small crystals of kaolinite; cells if present were obscured by fibrin	large mats of fibrin and mycelium with adherent kaolinite, very little free kaolinite

[a]2-layer, water: 65% (w/v) sucrose.
[b]Tryptone, yeast extract broth.
[c]Minimal salts medium.

mix the particulate well and remove it by centrifugation. The two samples were then tested for protein content, assuming the difference to be due to adsorption by the mineral particulate. The results obtained for kaolinite were reasonable. Fig. 1 contains a graph of the estimated protein content of dilutions of culture medium filtrate (CMF). Undiluted CMF contained about 65 μg of protein per ml. Addition of 1 mg/ml of kaolinite reduced the protein content to about 64 μg/ml and 2 mg/ml of kaolinite reduced it to about 55 μg/ml. Estimated protein concentrations were the average of three determinations. When bentonite was used as the absorbent, the apparent protein content was frequently higher than that of the original CMF and results were neither consistent from experiment to experiment, nor related linearly to protein or bentonite concentration. Fig. 1 includes a graph of one such determination, Fig. 3, another. Bentonite seemed to react in unpredictable ways with the reagents used for protein estimation. At higher concentrations, kaolinite seemed to increase the apparent protein conetnt also. In Fig. 2 and Fig. 3, the reduction of apparent protein content upon addition of 0.1 mg/ml of particulate is obvious, but so is the apparent increase of protein over the level of the control as increasing amounts of particulate were added. The sharp inflections of the curve in Fig. 3 at 40% CMF and 0.1 mg/ml of bentonite and at 20% CMF and 0.5 mg/ml of mineral are typical of the aberrant results obtained with bentonite. It is interesting to note in Fig. 3 that at concentrations of 1.0 mg/ml and 2.0 mg/ml of bentonite, the apparent increase of protein is essentially linear with increasing concentration of CMF and has the same slope as the control.

Although precise quantitation of protein adsprption to mineral particulates was not possible by this method, removal of protein from CMF and its presence on the mineral particualtes could be demonstrated by assaying for enzymatic activity. The assay was done on the same culture which provided the data for Figs. 1-3. The cells were resuspended in some of the CMF and homogenized, then removed by filtration.

The cells were suspended in buffer and diluted to an absorgance of 520 nm of 0.035. Four ml of cell suspensions were added aseptically to matched curvettes containing (o) no additions; (•) 3 mg of sterile Azocoll, or (+) 3 mg of sterile Axocoll and 10 mg of sterile kaolinite. Prior to each reading of absorbance, the curvettes were centrifuged to sediment the suspended particulates. Fig. 4 is a graph of absorbance due to release of chromophore from the Azocoll by the proteolytic activity of the cells in buffer. In 17 hr, the cell control (o) changed only slightly. By contrast, hydrolysis of Azocoll (•) increased the absorbance from 0.035 to 0.7. Addition of kaolinite may or may not have influenced enzyme activity. In curvettes containing kaolinite (+), released chromophore adhered to the mineral, making spectrophotometric measurement of released dye imprecise.

The proteolytic activity of CMF is illustrated in Fig. 5. The absorbance at 520 nm of CMF (o) was low and remained virtually constant throughout the 17 hr period. That CMF contained proteolytic enzymes is obvious from the curve of absorbance due to dye release (•). Even in the presence of kaolinite, which adsorbed some of the chromophore, adsorbance by released dye was considerable (+). To determine whether enzyme could be removed from CMF by kaolinite, the mineral (10 mg/ml) was added to CMF, the tubes were agitated for 5 min., then the suspended was centrifuged to remove the kaolinite. The supernatant was decanted and assayed. The pellet was resuspended in buffer and assayed for enzymatic activity. Fig. 6 shows the extent of dye release by the supernatant and a control sample of untreated CMF.

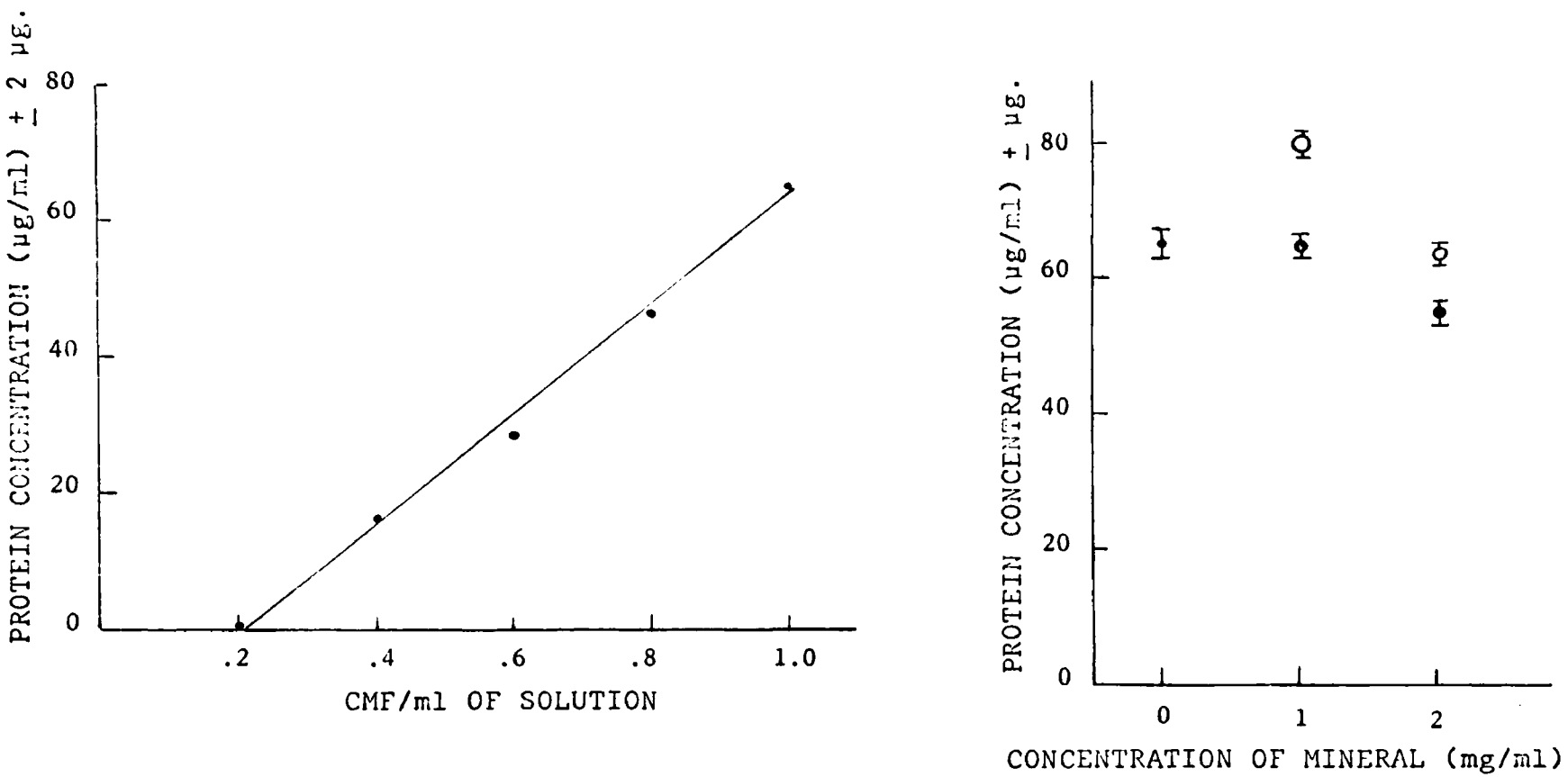

Fig. 1. -- Estimation by the Lowry method of protein in cell-free filtrates of chemically defined medium in which Micromonospora chalcea had been cultured. (a) Concentration of protein in medium diluted with buffer (●). (b) Concentration of protein remaining in medium after adsorption with kaolinite (o) or bentonite(●) at concentrations of 1 or 2 mg/ml.

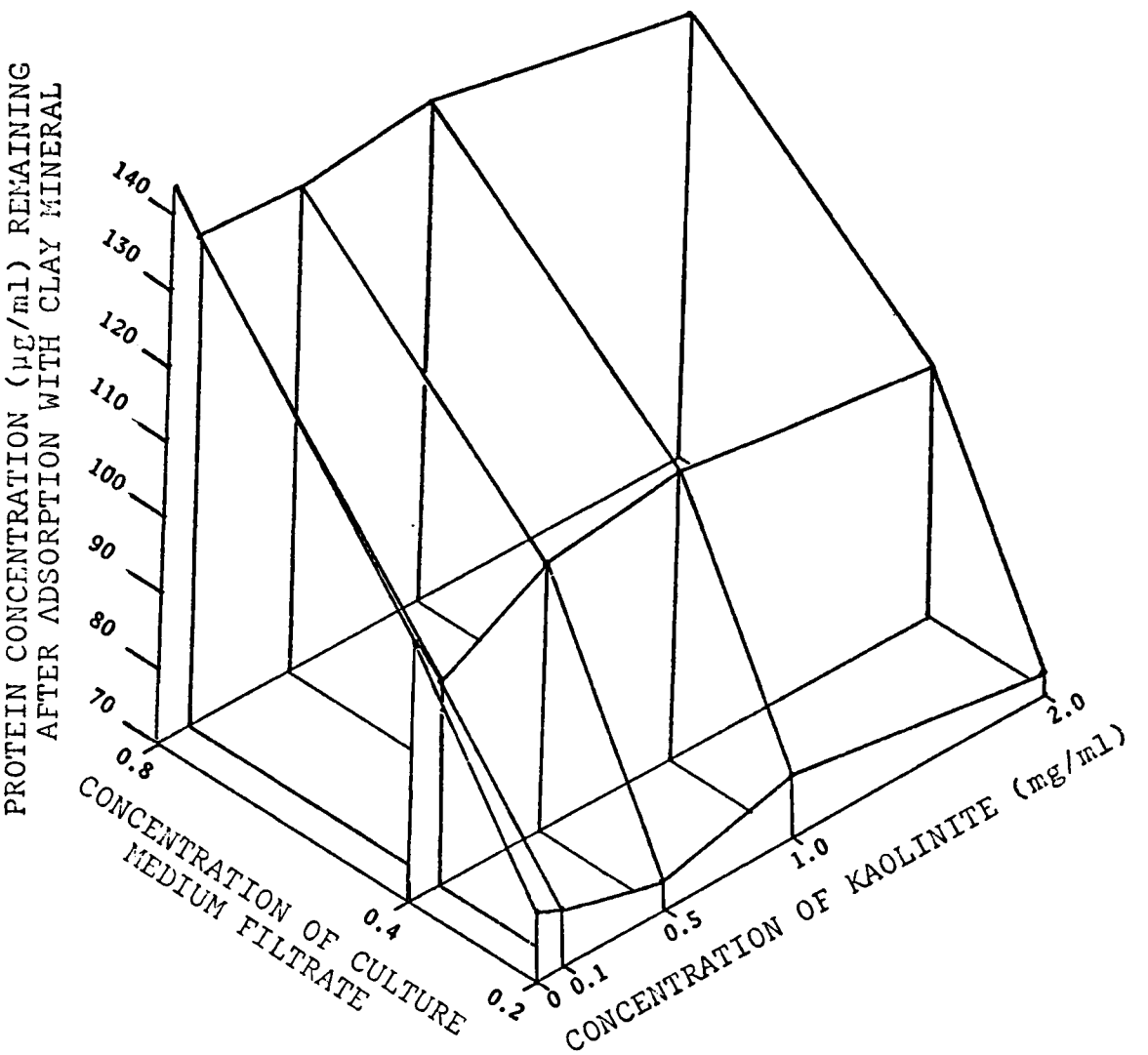

Fig. 2. -- Adsorption of protein by kaolinite. Dilutions of filtered medium in which Micromonospora chalcea had been cultures, were assayed after adsorption with 0, 0.1, 0.5, 1.0, and 2.0 mg/ml of kaolinite. Protein concentration was estimated by the Folin procedure (Lowry modification).

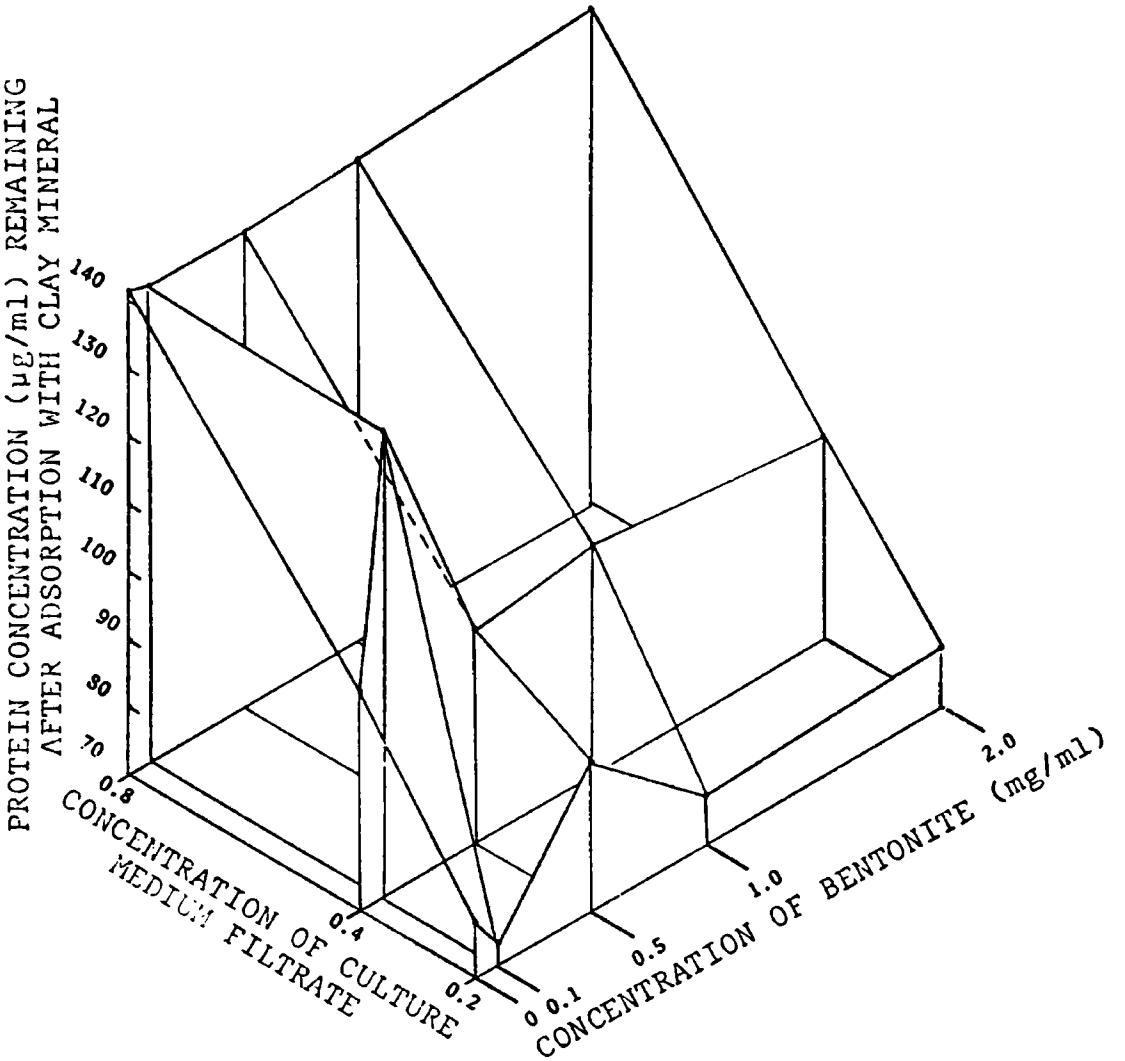

Fig. 3. -- Adsorption of protein by bentonite. Dilutions of filtered medium in which Micromonospora chalcea had been cultured, were assayed after adsorption with 0, 0. 1, 0. 5, 1. 0 and 2. 0 mg/ml of bentonite. Protein concentration was estimated by the Folin procedure (Lowry modification).

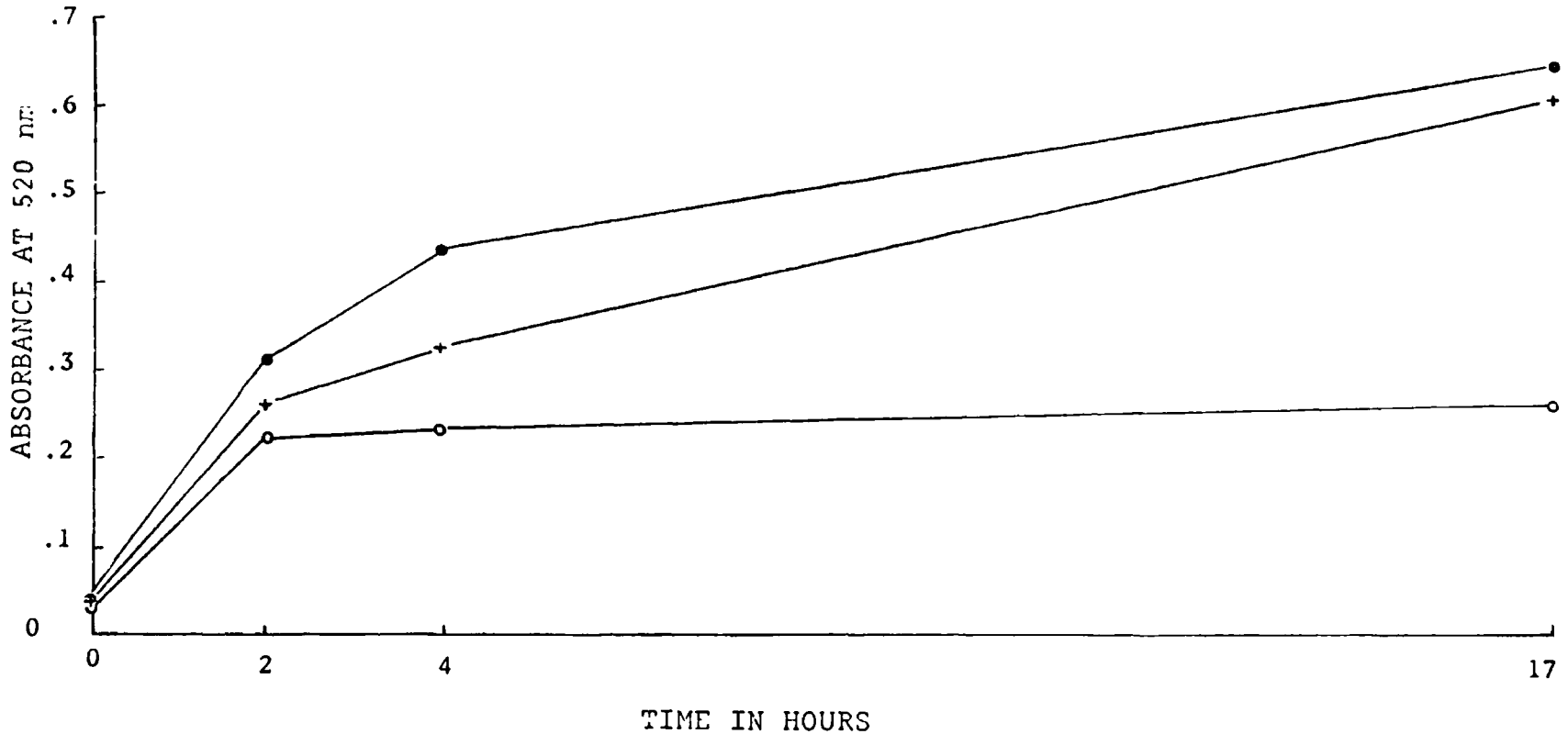

Fig. 4. -- Azocoll chromophore released by proteolytic activity by Micromonospora chalcea. Cell homogenate (absorbance at 520 nm = 0.035) was added to sterile matched curvettes containing: (o) no additions; (●) 3 mg of sterile Azocoll; (+) 3 mg of sterile Azocoll and 10 mg of sterile kaolinite. All curvettes were centrifuged to remove suspended particulates before absorbances were read at 520 nm.

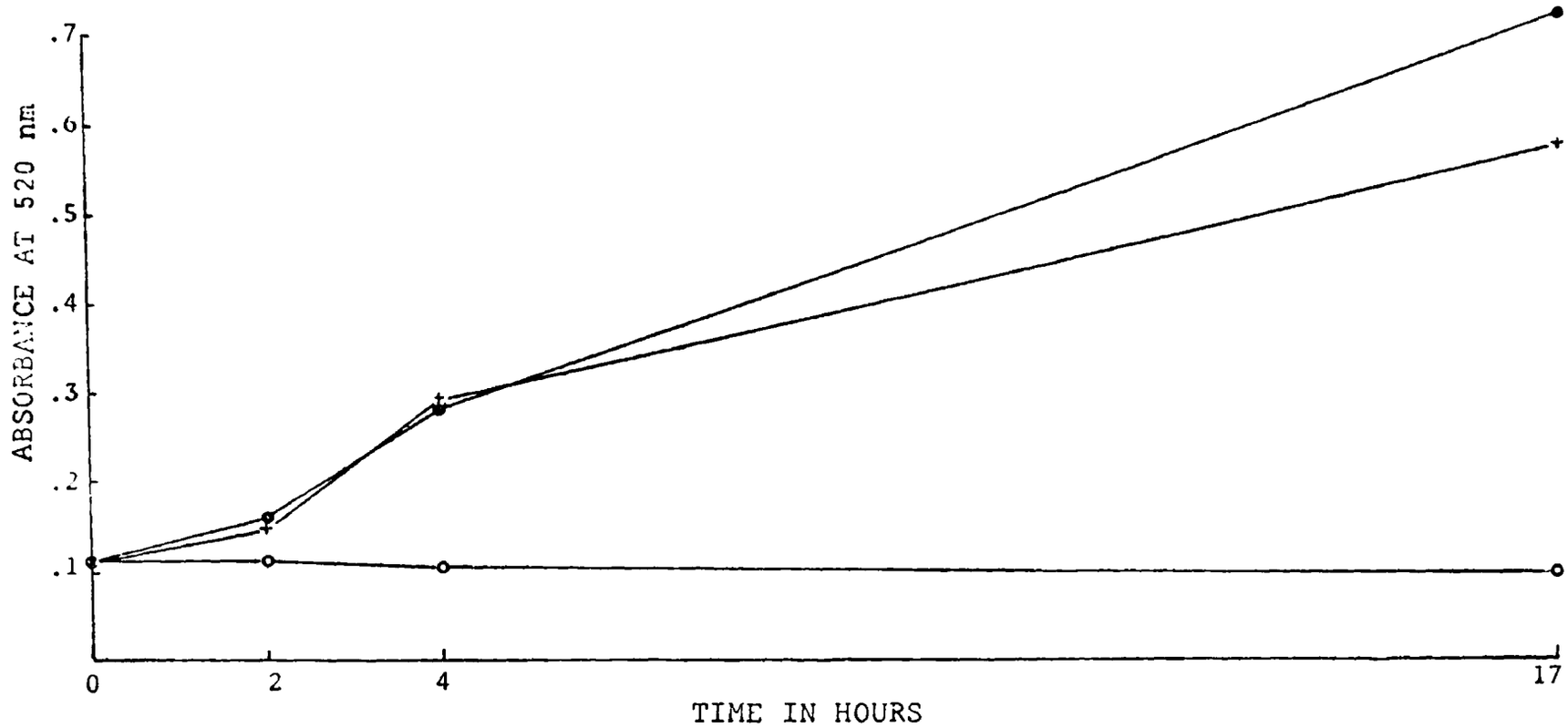

Fig. 5. -- Azocoll chromophore released by proteolytic activity of medium in which Micromonospora chalcea had been cultured and homogenized. Cells were removed by filtration. The filtrate was added to sterile, matched curvettes containing: (o) no additions; (●) 3 mg of sterile Azocoll; and (+) 3 mg of sterile Azocoll and 10 mg of sterile kaolinite. All curvettes were centrifuged to sediment suspended particles before absorbances were read at 520 nm.

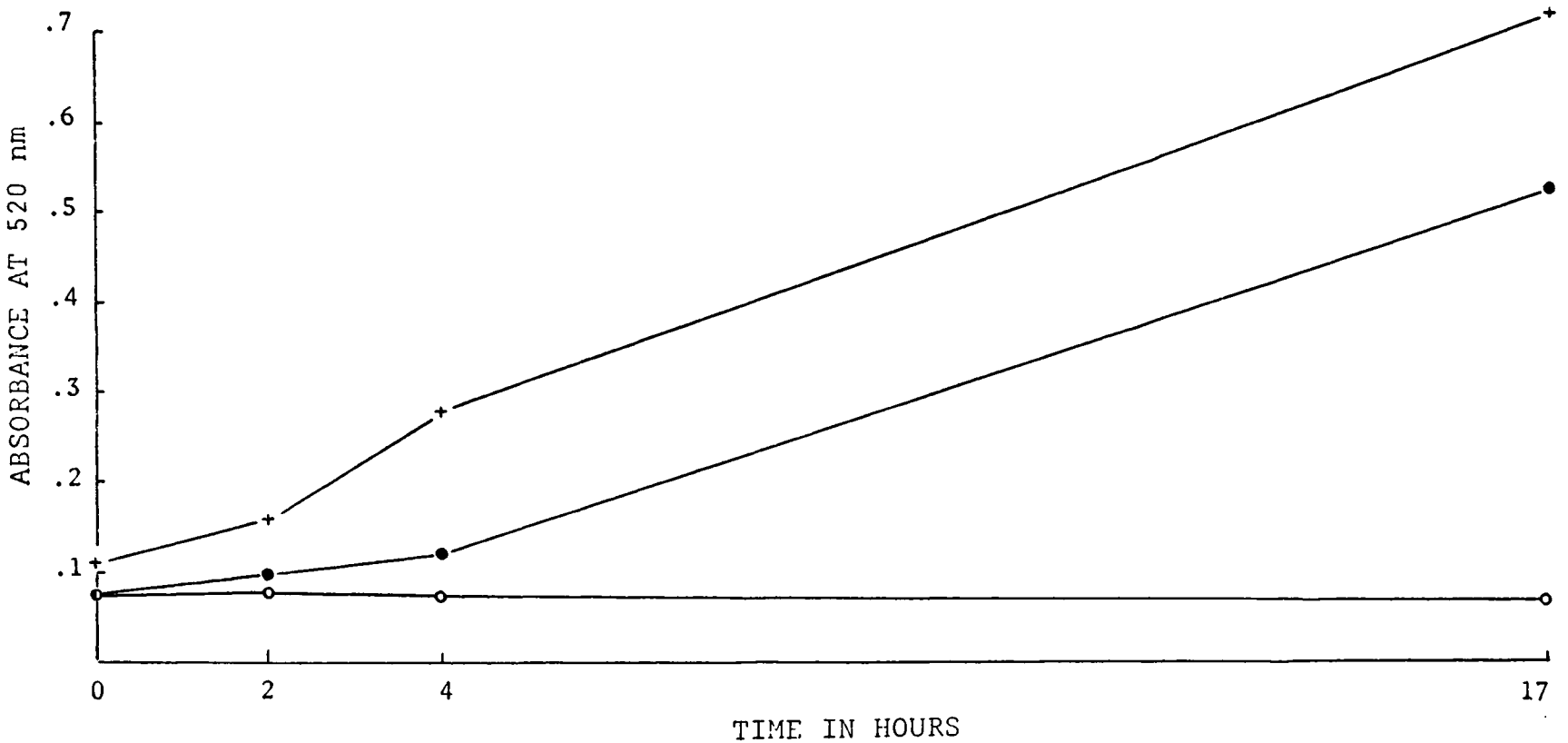

Fig. 6. -- Adsorption of enzyme by kaolinite. Medium in which Micromonospora chalcea had been cultured and homogenized, was filtered to remove cells. Kaolinite (10 mg/ml) was added to the cell-free filtrate, tubes were shaken for 5 minutes, then centrifuged to remove kaolinite. The supernatant fluid was added to sterile, matched curvettes containing: (o) no additions; and (●) 3 mg of Azocoll. As a control, cell-free filtrate to which kaolinite had not been added, was incubated with 3 mg of Azocoll (+). Absorbance at 520 nm of Azocoll chromophore released by residual enzymatic activity was measured after curvettes were centrifuged to remove suspended particles.

The supernatant, with no dye, had a low, constant level of absorbance (0). Dye release by the supernatant fluid (●) was considerable (reaching an absorbance of 0.5, after 17 hr) but was not so great as the activity of the intact CMF (+). The last, and most critical question to be asked in this experiment was whether enzyme absorbed by kaolinite from CMF were active. The kaolinite pellet removed from CMF was resuspended in buffer and measurement was made of absorbance due to the dye released (Fig. 7) by adsorbed enzyme. There was a marked release of chromophore, but its absorbance was diminished by the fact that kaolinite adsorbed the chromophore. The adsorbed chromophore could not be eluted from kaolinite (or bentonite) with 8M urea. No other elution procedures were attempted because a different experimental approach was used.

Degradation of Dye-Conjugated Collagen Substrates by Cell-Free Enzyme Preparations

The same procedure was used to prepare (1) control CMF; (2) CMF to which kaolinite or bentonite had been added, mixed, then removed by centrifugation; (3) and particulates removed from CMF and resuspended in buffer. To each of these solutions or suspensions, were added 0.25 mg/ml of HPA or Azocoll, the suspensions mixed well, 1 ml of 2% agar added at 47-50 C, the mixture agitated well, and the agar mixture poured into 15 mm x 55 mm petri plates.

Our original intention was simply to assay chromophore release macroscopically and microscopically by measuring the area of diffused dye surrounding the collagen particles. We found that mineral particulates exposed to CMF and resuspended in buffer adhered to HPA or Azocoll particles and effected decolorization or discolution of the collagen at a rate much greater than that of the original CMF. But dye diffusion could not be measured accurately in preparations containing mineral particulates. Again, the released chromophore adhered to the mineral particulates and was retained in the area of the collagen particle. However, we found that dissolution of the collagen and release of dye could be followed by time-photomicrography of selected collagen particles. Even after a mineral-coated collagen particle had been completely dissolved, its site was delineated by the mineral particles still trapped in the surrounding agar.

Table 3 lists the estimated total protein content (the same data as those graphed in Fig. 1) and enzymatic activity of the 9 preparations. Active enzyme was obviously located on the resuspended particulates; not in the CMF from which the particulates had been removed. More enzyme activity was associated with bentonite than with kaolinite. This could be due either to bentonite's smaller particle size, hence more surface area per mg of material, or to its greater surface charge and exchange capacity.

Isolation of Streptomyces and Micromonospora Strains from Lake Erie

To compare reactions with mineral particulates and enzyme activity of recently isolated actinomycetes with the results obtained with laboratory strains, samples of surface or bottom water and mud from Lake Erie were used to isolate streptomycetes and micromonosporas. To discourage vegetative cells of bacteria and select for the more heat resistant

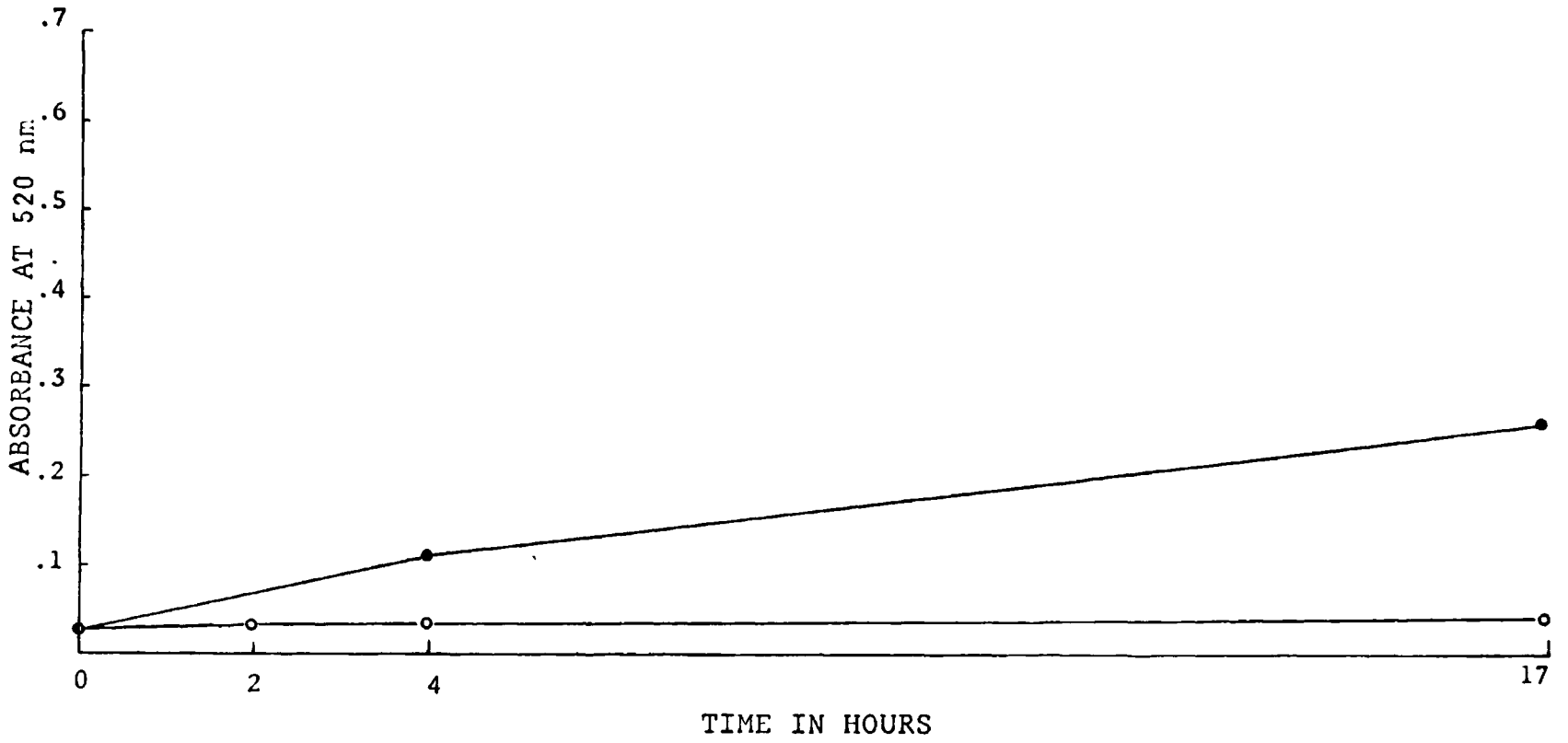

Fig. 7. -- Azocoll chromophore released by proteolytic enzyme adsorbed to kaolinite. Kaolinite was shaken with and removed by centrifugation from cell-free filtrate of medium in which Micromonospora chalcea had been cultured and homogenized. The kaolinite was resuspended in 0.01M Tris buffer at pH 7.6, and added to sterile, matched curvettes containing : (o) no additions; (●) 3 mg of sterile Azocoll.

TABLE 3

Enzyme activity, estimated by rate and extent of chromophore release from Azocoll, (1) in culture medium filtrate (CMF_1) from Micromonospora chalcea cultured 3 days in chemically defined meduim; (2) on particulates added to CMF_1, removed by centrifugation and resuspended in Tris buffer; and (3) in supernatant fluid (CMF_2) after addition and removal of mineral particulates. Azocoll + sample were poured in agar.

Sample	Mineral Particulate	Concentration of Mineral	Total Protein Concentration of Fluid[a]	Chromophore Release from Azocoll	
				48 hours	72 hours
CMF_1	None	0	65 ± 2 μg	NC[b]	Slight diffusion of dye
Buffer + Mineral	Kaolinite	2 mg/ml	ND[c]	Partial decolorization of some particles	Partial decolorization of most particles
		1 mg/ml	ND	Considerable dye release	≥ 50% of particles completely decolorized
	Bentonite	2 mg/ml	ND	≥ 50% of particles completely decolorized	Azocoll completely dissolved; only mineral outlines remained[d]
		1 mg/ml	ND	≥ 50% of particles completely decolorized	Azocoll completely dissolved; only mineral outlines remained
CMF_2	Kaolinite	2 mg/ml	63 ± 1 μg	NC	Some diffusion of dye
		1 mg/ml	79 ± 2 μg	NC	Some diffusion of dye
	Bentonite	2 mg/ml	55 ± 2 μg	NC	Little, if any, change
		1 mg/ml	64 ± 2 μg	NC	Little, if any, change

[a]Estimated by Lowry method [b]No change. [c]Not determined. [d]delineating sites of Azocoll in the agar.

mycelia and spores of actinomyces, water samples were incubated at 70 C for various periods of time. Table 4 lists the strains isolated; the media (mostly selective for actinomycetes) used; and the concentrations of Rose Bengal included in media to inhibit fungi and bacteria. All individual actinomycete colonies were picked for isolation. In no case were any two colonies apparently of the same strain of organism. Because of an error in the collecting procedure, trichloroacetic acid was added to the water sample collected on 7/2/71. On the 24 plates streaked from that water sample, only one colony developed, the isolate designed S-3. This indicent indicates the durability of this strain of Streptomyces, or the effectiveness of trichloroacetic acid in inhibiting microbial metabolism. The precursor of the S-3 colony had been to contact with the trichloroacetic acid solution for more than 3 hours before the water sample was streaked on the YME plate.

Studies of Organisms Isolated from Lake Erie

Screening of Isolated Actinomycetes for Culture Characteristics and Substrate Utilization

We wished to work with an organism which could utilize a wide spectrum of substrates, and which would culture rapidly in chemically defined medium, with or without addition of amino acids. Three strains of Streptomyces and 4 of Micromonospora were screened for their ability to grow on a variety of media. Table 5 indicates the substrate utilization by all strains isolated. Fig. 8 elaborates on the growth in GM of the 3 most active strains relative to their growth in TYE. One streptomycete and 2 micromonosporas developed rapidly in all complex media. We chose to work with the isolate of streptomycete designated S-1, because it could be transferred directly from YME to chemically defined media. The micromonosporas required culture in TYE prior to transfer to GM.

Further Studies of Substrate Utilization by Streptomyces sp. S-1

Utilization of Collagen

Because there has been some controversy (Mandel, 1961) as to whether streptomycetes produce a trye collagenase enzyme, the collagen preparation being used for trypsin sensitivity was tested before and afrer autoclaving the collagen. Before autoclaving, a sample of collagen resisted tryptic digestion for 5 days at 27 C. A replicate sample, autoclaved, was completely dissolved in 3 hours. Because it has been reported that Ca^{++} is necessary for Clostridium histolyticum collagenase activity (26), 0.01M $CaCl_2$ was added to MSM (designated MSM-$CaCl_2$). Collagen was added to flasks of both MSM and MSM-$CaCl_2$, and all the flasks of media were autoclaved. After flasks of MSM and MSM-$CaCl_2$ without collagen had cooled, unautoclaved collagen was added, and all flasks were inoculated with S-1. One flask each of MSM and MSM-$CaCl_2$, contained pure cultures of S-1 (determined by subcultures streaked on YME plates). Whether collagen had been autoclaved or not, growth of S-1 was better in media containing $CaCl_2$. Figs. 9b, 15a and b, and Fig. 30 illustrate growth of culture S-1 in MSM containing collagen.

TABLE 4

Isolation of Actinomycetes from surface or bottom water and mud from the Western Basin of Lake Erie

Organism designated:	OSU#	Date of sample collection	Location	Incubation time (min) at 70 C	Medium on which colony developed	Concentration of Rose Bengal
Streptomyces sp.						
S-1	823	6/19/70	bottom mud 15 M depth	0	AIA[a]	1:20,000
S-2	824	6/19/70	"	10	JEN[b]	1:30,000
S-3	827	7/2/70	surface water	0	YME[c]	0
Micromonospora sp.						
M-1	825	6/19/70	bottom mud 15 M depth	10	AIA	1:20,000
M-2	826	6/19/70	"	20	AIA	1:40,000
M-3	804	6/19/70	"	20	JEN	1:30,000
M-4	805	6/19/70	"	20	JEN	1:30,000

[a]Actinomycete isolation agar
[b]Jensen's starch, casein agar
[c]Yeast extract agar

TABLE 5

Substrate utilization by actinomycetes isolated from Lake Erie

Substrates	Organisms: Streptomyces strains			Micromonospora strains			
	S-1	S-2	S-3	M-1	M-2	M-3	M-4
Actinomycete isolation agar	+[a]	+	+	+[a]	+[a]	NT[b]	NT[b]
Jensen's agar	NT[b]	+[a]	+	NT[b]	NT[b]	+[a]	+[a]
Yeast extract, malt extract agar (YME)	+	+	+[a]	+	+	+	+
Tryptone, yeast extract agar	+	+	+	+	+	+	+
GM[c] inoculated from washed cells	+	-	-	+	+	+	NT[d]
GM inoculated from YME slants	+	-	-	-	-	-	±[e]
MSM[f] + chitin	+	-	-	+	+	+	±
MSM + denatured collagen	+	-	-	+	+	±	+
MSM + casein	+	NT[b]	NT[b]	+	+	NT[b]	NT[b]
MSM + cellulose + CH_3COONH_4	+	-	-	+	+	±	NT[d]

[a] Medium on which organism was isolated.

[b] Not tested because ability to use that substrate had been demonstrated on another medium.

[c] Minimal salts medium + glucose + asparagine + CH_3COONH_4.

[d] Not tested because organism was difficult to culture

[e] Very slight growth.

[f] Mineral salts medium.

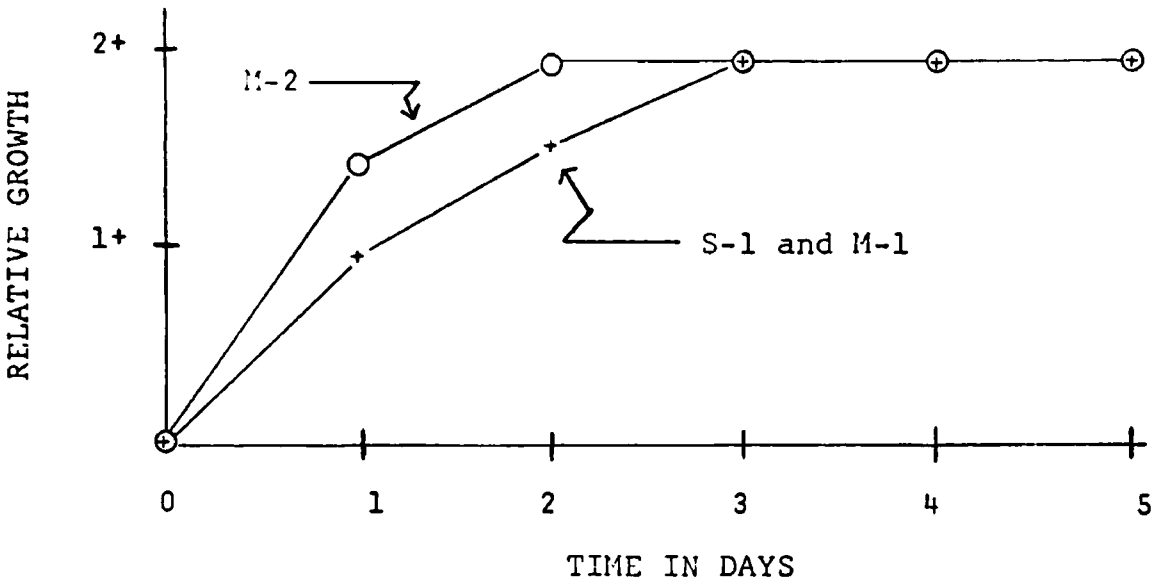

Fig. 8. -- Comparison of growth rates of 3 Actinomyces isolated from Lake Erie. The three strains, Streptomyces sp. S-1, Micromonospora sp. M-1, and Micromonospora sp. M-2, were tested for their ability to culture in a chemically defined medium containing minimal salts medium, glucose, asparagine and CH_3COONH_4.

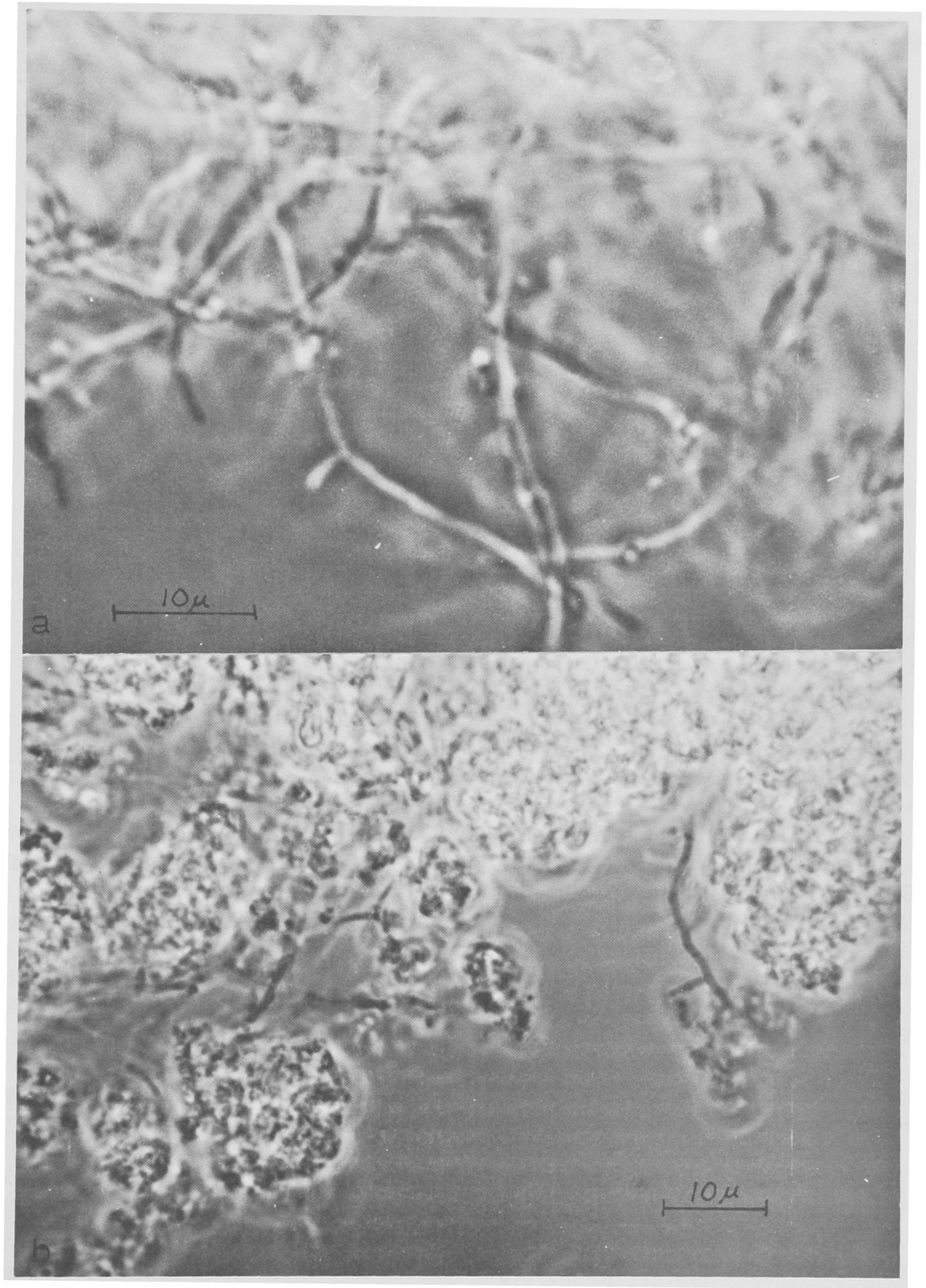

Fig. 9. -- Comparison of Streptomyces sp. S-1 cultured in (a) chemically defined medium containing gluxose, asparagine and CH_3COONH_4; and (b) minimal salts medium containing 0.01M $CaCl_2$ and native collagen.

Utilization of Chitin

The growth of S-1 in MSM + chitin was extremely rapid. A 24 hr culture of S-1 with chitin as sole carbon and nitrogen source was equivalent to a 48 hr culture of S-1 in the complex organic medium, TYE. Fig. 10 shows flasks of 26 hr cultures of S-1 in MSM-2TB; MSM-SO_4-2TB + glucose (4 mg/ml); MSM + chitin (4 mg/ml); and MSM + chitin (4 mg/ml) + kaolinite (1 mg/ml). Fig. 11 is a composite of micrographs of mycelia from the 4 cultures. Because the chitin preparation was not necessarily free of other types of organic material, it was possible that some growth promoting material was present as a contaminant. Cralley (1968) had tested samples from the same chitin preparation for soluble carbohydrates (anthrone procedure) and amino compounds (ninhydrin procedure). Amounts < 0.14 μg of soluble amino compounds per gram of chitin were found. Since such low levels of nutrients would not account for the tremendous growth of S-1 in chitin media, a test was run for a trace material which might serve as a growth promoting substance.

Test for growth promoting factor associated with chitin:

To determine whether there were a growth promoting factor in MSM + chitin medium, which was (1) soluble in the medium, or (2) retained on the chitin through several days of incubation on the rotary shaker at 27 C; and if soluble, if there were (3) able to promote growth in adequate media, or (4) able to substitute for various nutrients in deficient media, the following experiment was conducted.

Chitin (0.2 g) was placed in each of two flasks (A and B) each containing 100 ml of MSM. These were autoclaved, cooled, and placed on the rotary shaker at 27 C for 7 days. Then the chitin was removed from flask A by centrifugation of the contents for 15 min at 12,000 x g. The supernatant fluid was filtered through a Millipore filter (pore size, 100 μm) and 5 ml of filtrate was added to 1 each of flasks containing 100 ml of one of the following media: (a) MSM-2TB; (b) MSM-2TB-SO_4; (c) MSM-2TB + glucose (4 g/liter); (d) MSM-2TB-SO_4 + glucose (4 g/liter) and (e) GM. From each of the flasks to which filtrate from flask A had been added, a 5 ml sample was removed aseptically and placed in a separate sterile culture tube. From each of the flasks containing media (a) - (e) to which filtrate from flask A had not been added, a 5 ml sample was removed in the same way. Washed S-1 cells from a GM culture were homogenized. A standard inoculum of 1 ml was added to each of the flasks containing media (a) - (e), with and without filtrate from flask A. Flask B and a flask (C) of MSM to which the chitin removed from flask A was added, were also inoculated. The 10 culture tubes were each inoculated with a loop of Escherichia coli (strain 430) from a 19 hr GM culture. These tubes were incubated for 7 days at 37 C, with daily checks on turbidity.

There was no grossly observable difference of rate or of total amount of growth, of either S-1 or E. coli in media with and without the filtrate from flask A. There was no difference between the rate or amount of growth of S-1 cells in flasks B and C. If a growth promoting factor were present in the crude chitin, either it was not soluble, or greater quantities would be required to substitute for carbon or nitrogen in the deficient media. The fact that E. coli did not respond at all to addition of the filtrate to its media proves

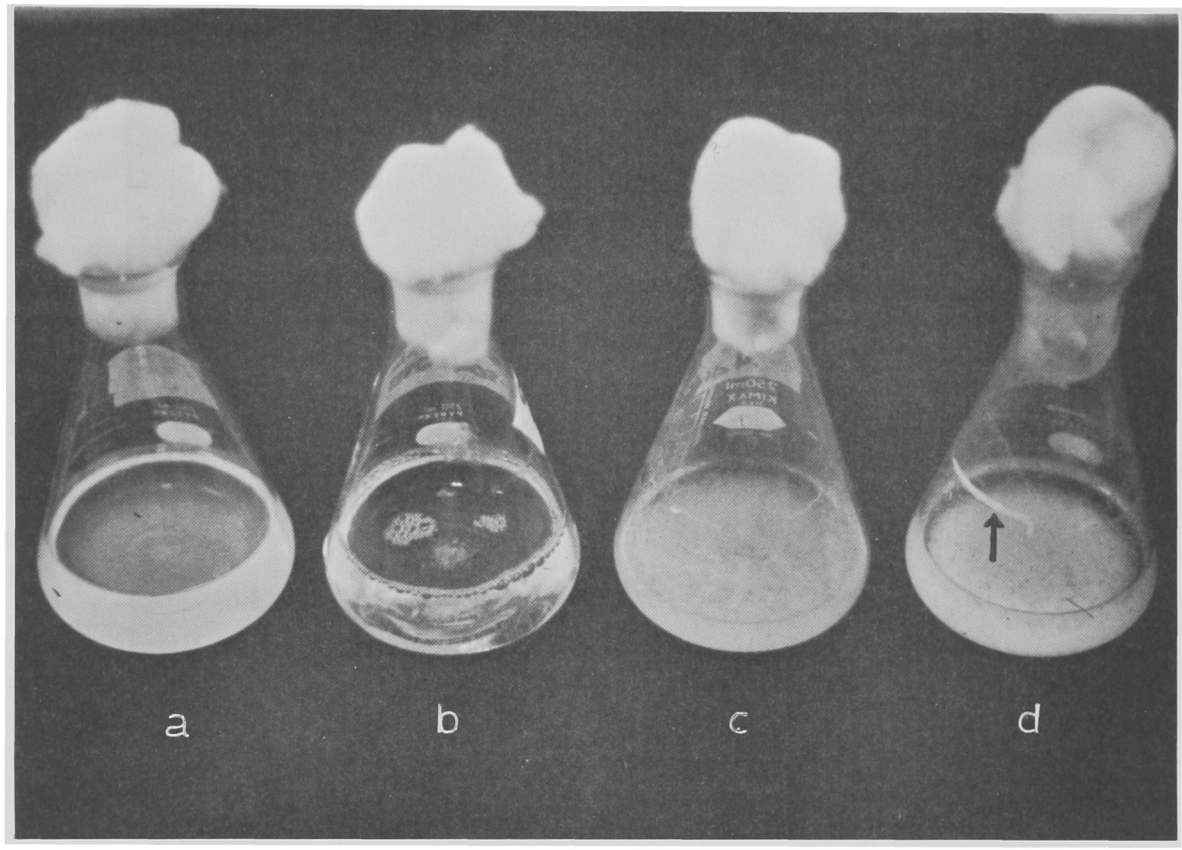

Fig. 10. -- Macroscopic appearance of Streptomyces sp. S-1 cultured in minimal salts medium (MSM); MSM + glucose + NH_4SO_4; MSM + chitin, and MSM + chitin + kaolinite.

All Flasks contained 26 hr cultures of standard inoculum. (a) S-1 cells in minimal salts medium with 0. 002M Tris buffer; no carbon or nitrigen source was added. The cloudy appearance of the medium was due to precipitation of mineral salts in the absence of cell metabolism. (b) S-1 cells in minimal salts medium with 0. 002M Tris buffer; $(NH_4)_2SO_4$, 2. 64 mg/ml; and glucose, 4 mg/ml. Little growth had occurred, but cells had not autolysed. Slight frothing of the medium apparently was due to production of extracellular polymers by the streptomycete. Note the absence of mineral precipitate. (c) S-1 cells in mineral salts medium with 0,002M Tris buffer and 4 mg/ml of chitin. Growth was very heaby. (d) S-1 cells with medium as in (c) to which kaolinite (1 mg/ml) was added. Note the ring of cells-chitin-kaolinite-aggregate adhering to the flask wall (arrow) at the fluid level reached by the medium while the flask was on the rotary shaker.

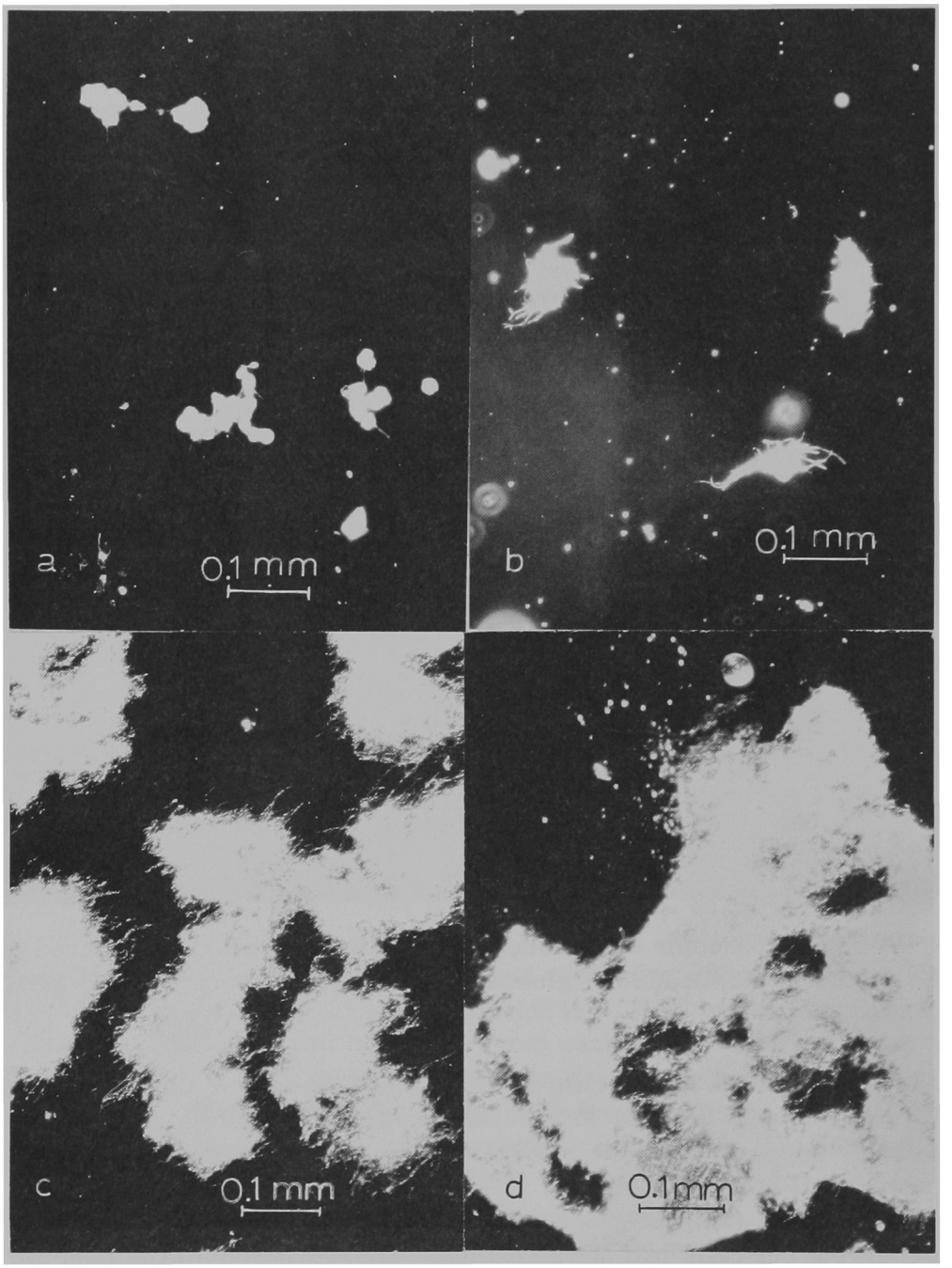

Fig. 11. -- Microscopic appearance of Streptomyces sp. S-1 cultured in minimal salts medium (MSM); MSM + glucose + NH_4SO_4; MSM + chitin + kaolinite.

All flasks contained 26 hr cultures of a standard inoculum. (a) S)2 cells in minimal salts medium with 0. 002M Tris buffer, with no carbon or nitrogen source added. the rounded appearance of the cells is typical of autolysis. (b) S-1 cells in minimal salts medium with 0. 002M Tris buffer; $(NH_4)_2SO_4$, 2. 64 mg/ml; and glucose, 4 mg/ml. Little growth had occurred, but cells had not autolysed as in (a). (c) S-1 cells in mineral salts medium with 0. 002M Tris buffer and 4 mg/ml of chitin. Growth was very heavy. (d) S-1 cells with medium as in (c) to which kaolinite (1 mg/ml) was added.

only that no metabolite useful to it was present.

Utilization of Cellulose

In cultures of S-1 in MSM-SO_4-2TB + 4 gm/liter of cellulose, cell mass increased very slowly, but autolysis did not occur. However, S-1 inoculated into MSM-SO_4-2TB containing kaolinite (1 mg/ml) did not autolyze either. In both cases, S-1 could be seen aggregated with the particulate. To insure that S-1 was utilizing cellulose as a carbon source, not merely surviving in its presence, utilization of cellobiose by S-1, and the ability of S-1 to hydrolyze cellulose in agar were tested.

Triplicate flasks of MSM-SO_4-2TB + glucose (1 g/liter) and MSM-SO_4-2TB + cellobiose (1 g/liter) were inoculated with a standard inoculum of S-1. After 14 days, cells were removed from the cultures by centrifugation and dry weights of cell pellets were determined. Statistical analysis of dry weights indicated no significant difference at the 1 % level between cell masses of glucose and cellobiose cultures.

Cellulose agar plates were prepared with agar about 5 mm deep. As the agar cooled, the cellulose settled to the bottom. The agar appeared opaque due to the layer of white cellulose particles.

S-1 was streaked onto the plates from the following media: TYE; MSM-2TB; MSM-SO_4-2TB + glucose, and MSM-SO_4-2TB + cellobiose. The plates were placed over moist cotton in containers covered with parafilm and incubated at 27 C. Water was added to the cotton when necessary. The plates were examined daily for 2 weeks. There was no hydrolysis of cellulose visible macroscopically or microscopically, but surface colonies of S-1 grew and spread, even on the plate streaked with S-1 in MSM. After 6 weeks, plates were examined again; all 4 plates showed hydrolysis of cellulose. Plates streaked with S-1 cultured in MSM-SO_4-2TB + cellobiose and in MSM-SO_4-2TB + glucose showed the most pronounced cellulose degradation. Macroscopically, the agar in those two plates appeared mottled, with patches of clear and opaque agar. The other two plates still appeared opaque, but microscopic examination revealed changes in the cellulose. Fig. 12a shows undegraded cellulose from an uninoculated area of a plate. Figs. 12b, 13, and 14 show progressive growth of S-1 in the area originally occupied by cellulose. Figs. 13 and 14 were photographed from areas in which the agar appeared clear; Fig. 12b from the boundary of clear (c) and opaque (o) areas. Figs. 12 to 14 are of S-1 streaked from the cellobiose culture. The plate streaked with S-1 from TYE showed only slight penetration of mycelia into the base of the agar, where the cellulose was. Degradation of cellulose had advanced to an intermediate degree in the plate streaked with S-1 in MSM.

The mycelium of S-1 cultures had different appearances in different media:

The mycelium of S-1 cultures had different appearances in different media. We did not investigate the causes of these differences but have recorded them photographically. Fig. 9 compares (a) the mycelium of S-1 cultured in GM with (b) that of S-1 cultured in MSM-$CaCl_2$ containing autoclaved collagen (0.05 g/100 ml). The unidentified material clustered over the mycelium could be seen in collagen cultures of S-1 in MSM with or without

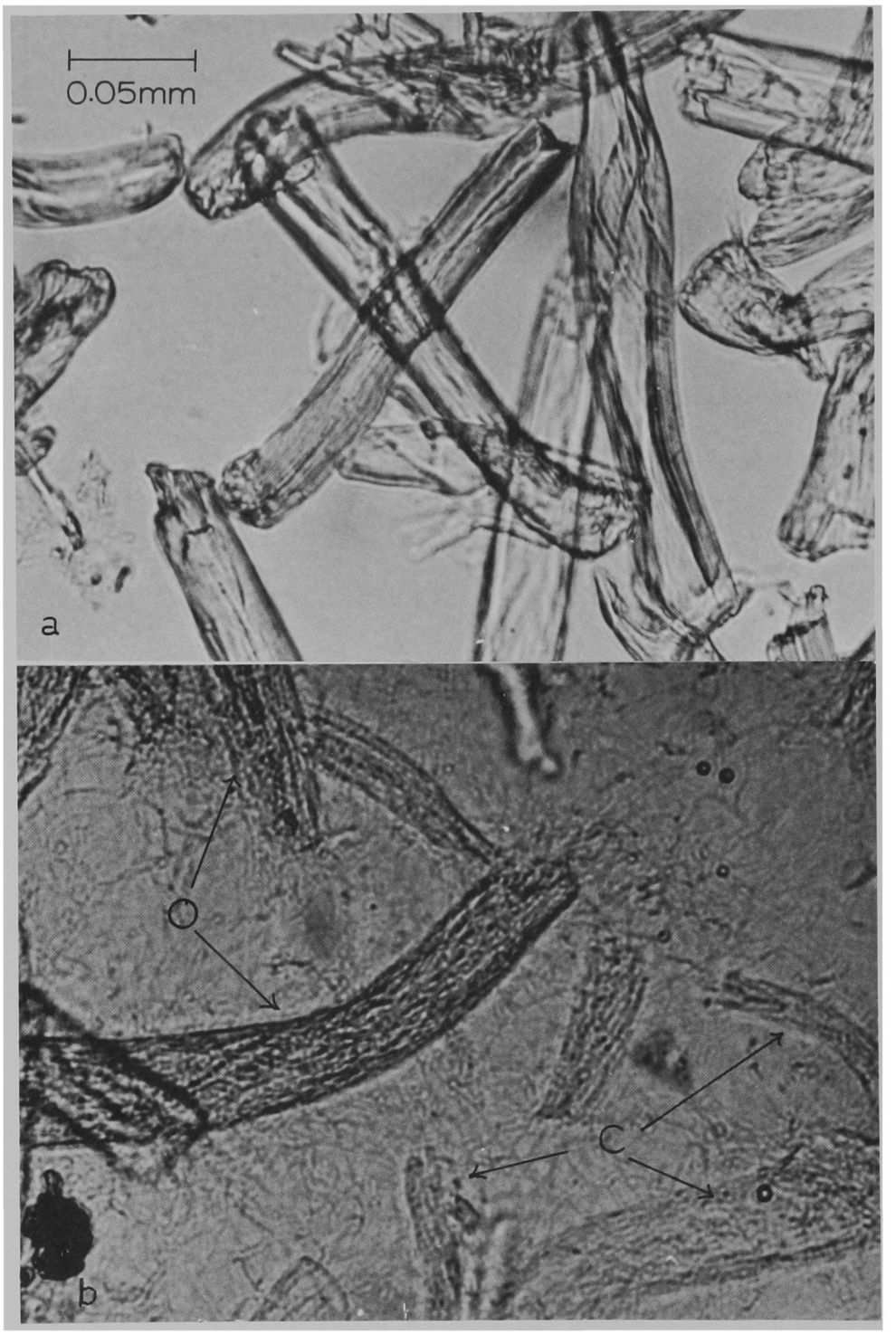

Fig. 12. -- Hydrolysis of cellulose by Streptomyces sp. S-1. Microscopic appearance of cellulose agar plates: (a) shows an area where hydrolysis had not occurred. Macroscopically this area of the plate was opaque due to the suspended cellulose fibers. (b) is an area of the plate where partial hydrolysis had occurred; mycelia had influtrated the area, some within the fibrils. Macroscopically this area of the plate appeared mottled. The area designated (c) was clear; the area (o) was opaque. (440X)

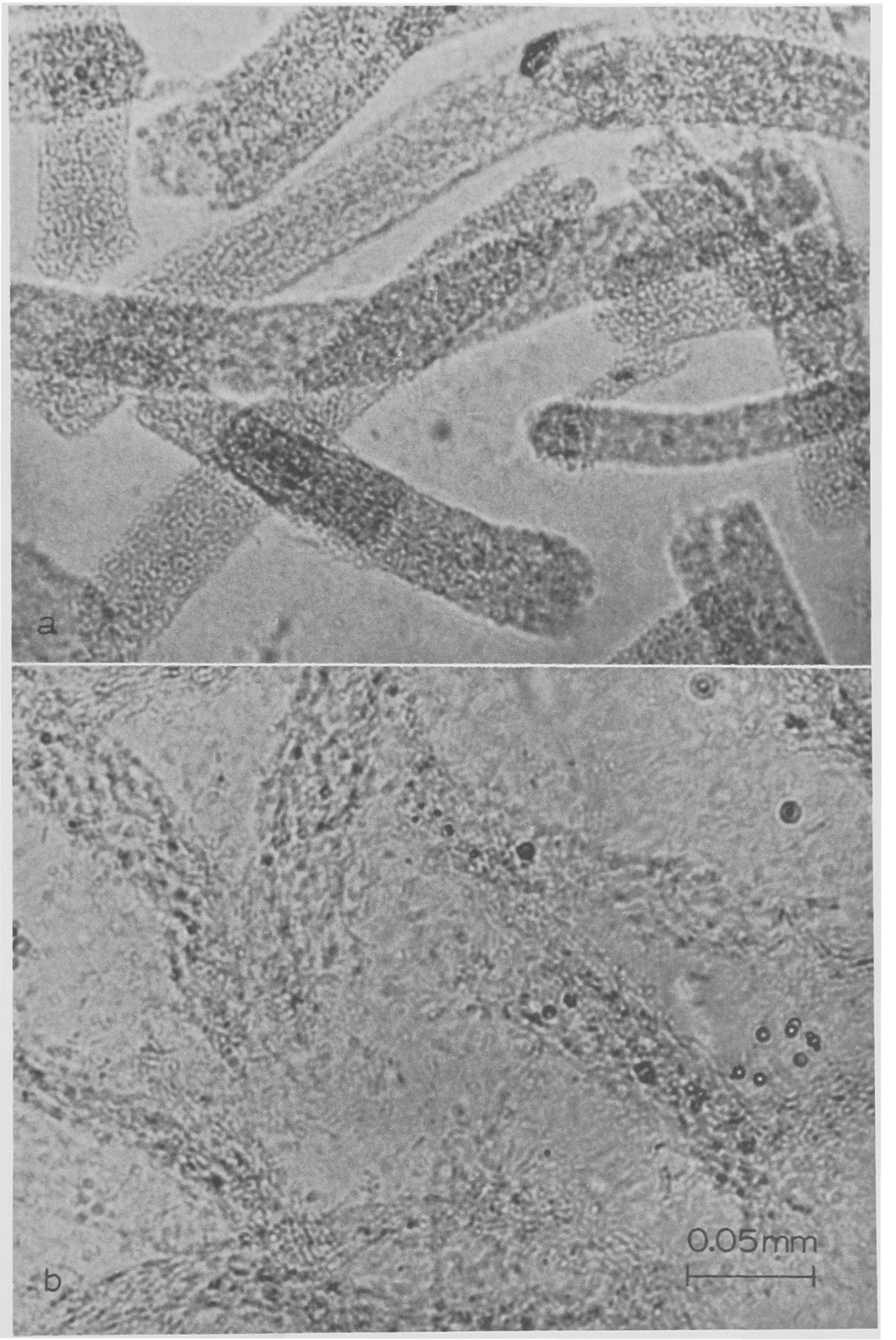

Fig. 13. -- Dissolution of cellulose by Streptomyces sp. S-1. (a) Mycelia had influtrated the area originally occupied by cellulose in the agar. No fibrillar cellulose structure remained, but the streptomycete seemed to have been confined to intrafibrillar areas. (b) Mycelia were not confined to the area originally occupied by the cellulose. Macroscopically, both areas of the plate appeared clear. (400X)

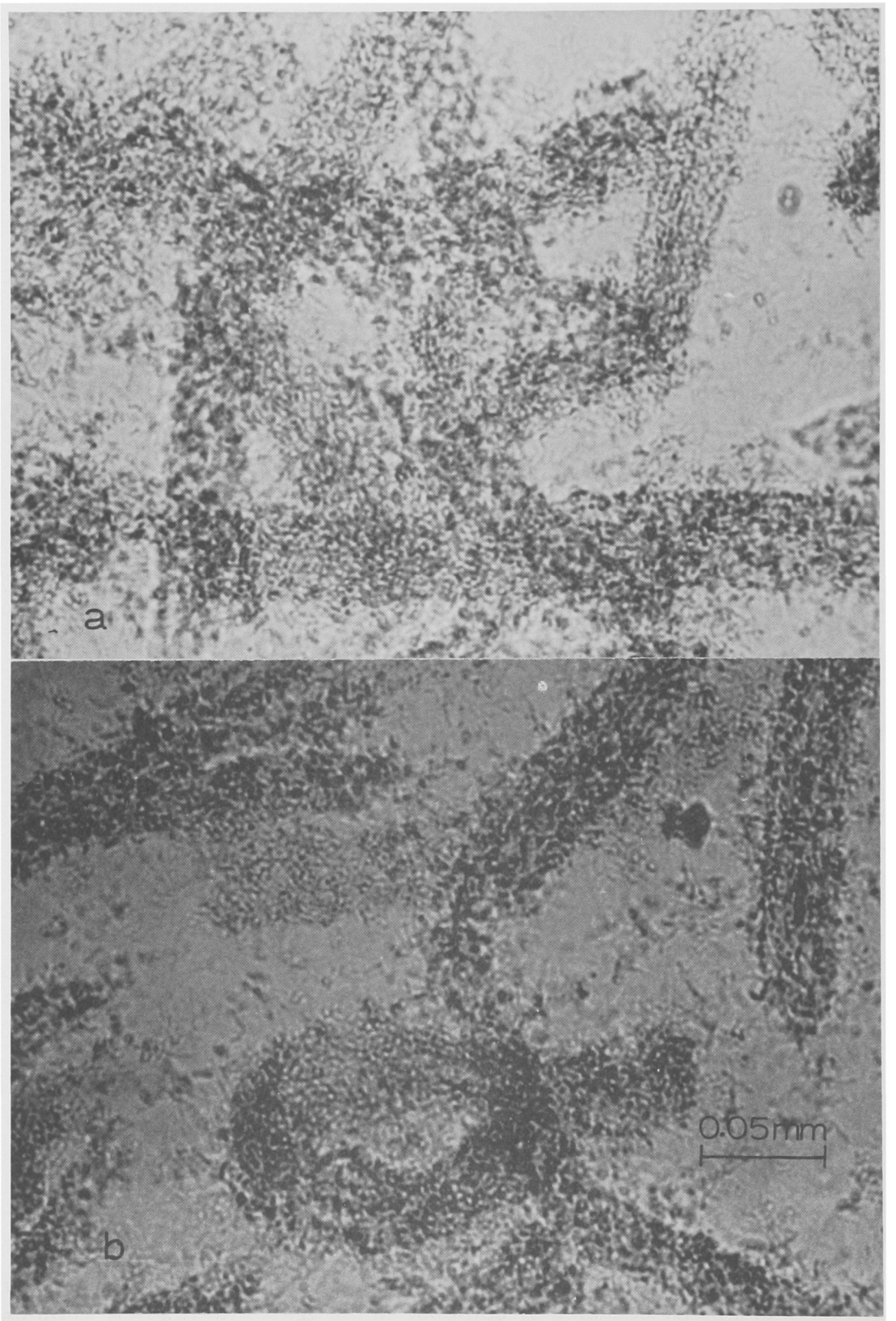

Fig. 14. -- Growth of Streptomyces sp. S-1 in cellulose agar after complete dissolution of the cellulose. (a) and (b) illustrate progressive growth of the mycelia in areas originally occupied by cellulose fibers. (400X)

$CaCl_2$, and with native or autoclaved collagen (see Fig. 15). But the material was most dense in cultures of S-1 in MSM-$CaCl_2$ + native collagen. Fig. 15 contrasts the appearance of (a) a 5 day culture of S-1 in MSM-$CaCl_2$ + native collagen; (b) a 3 day culture of S-1 in MSM + autoclaved collagen; about 10% of the colonies in that culture had assumed the ringed form pictured; and (c) a 3 day culture of S-1 in MSM + chitin. The fluffy, reflective appearance of the colony pictured was typical of the whole culture.

Adherence of Mineral Particulates to Cells

Three procedures were used to demonstrate qualitatively and quantitatively that mineral particulates adhere to S-1 mycelium: photography, comparison of sedimentation velocities of cells, particulates, and mixtures of the two; and density gradient centrifugation on gradients which would separate mycelia from particulates unless a strong binding force were responsible for their adherence.

Photography

Effect of pH at constant ionic strength:

A culture of S-1 cells was homogenized and divided into 6 samples. The pH of one sample (8.2) was recorded, those of the other five samples were adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0. Each of these samples was divided into 3 portions and a mineral particulate, kaolinite, illite or bentonite was added to one tube at each pH. No particulate was added to the sample at pH 8.2. The tubes were agitated well to mix the contents, pH's were rechecked and adjusted again where necessary, and distilled water was added to equalize total volumes. Samples were withdrawn by Pasteur pipette for preparation of wet mounts, which were photographed. Fig. 16 shows the appearance of S-1 mycelia at various pH's after addition of kaolinite. Figs. 17 and 18 show S-1 mycelia under the same conditions with addition of illite and bentonite respectively.

In all cases, mineral particulates adhered to mycelia at all pH's tested. Optimal adsorption of kaolinite to cells seemed to occur at pH's 6 and 7. At higher pH's there was more free kaolinite present in the culture medium. This could be expected since kaolinite carries a net negative charge, and as pH increases, the cell surfaces would become progressively more anionic. That attraction between mineral and cells occurs at higher pH's is obvious from the photographs. This may be explained by McLaren's (51) observation that the actual surface pH of kaolinite is 2 pH units lower than the pH measured in the surrounding solution. Thus, the effective pH's of the cell-kaolinite interfaces in the preparations photographed may have ranged from 4 to 8, not 6 to 10.

Optimal adherence of illite to streptomycete mycelia seemed to occur at pH 8, but at all pH's tested the mycelial clusters were heavily coated with mineral. By contrast, at all pH's, much bentonite was free in the culture fluid and only at pH 9 did the mycelium seem to be densely coated with mineral. Some of the difference in appearance of cell-bentonite aggregates may be due to the difference in particle sizes of the mineral. Individual particles of bentonite are so small as to be almost indistinguishable from mycelium at the magnification used. Density gradient centrifugation of cells cultured with bentonite in the

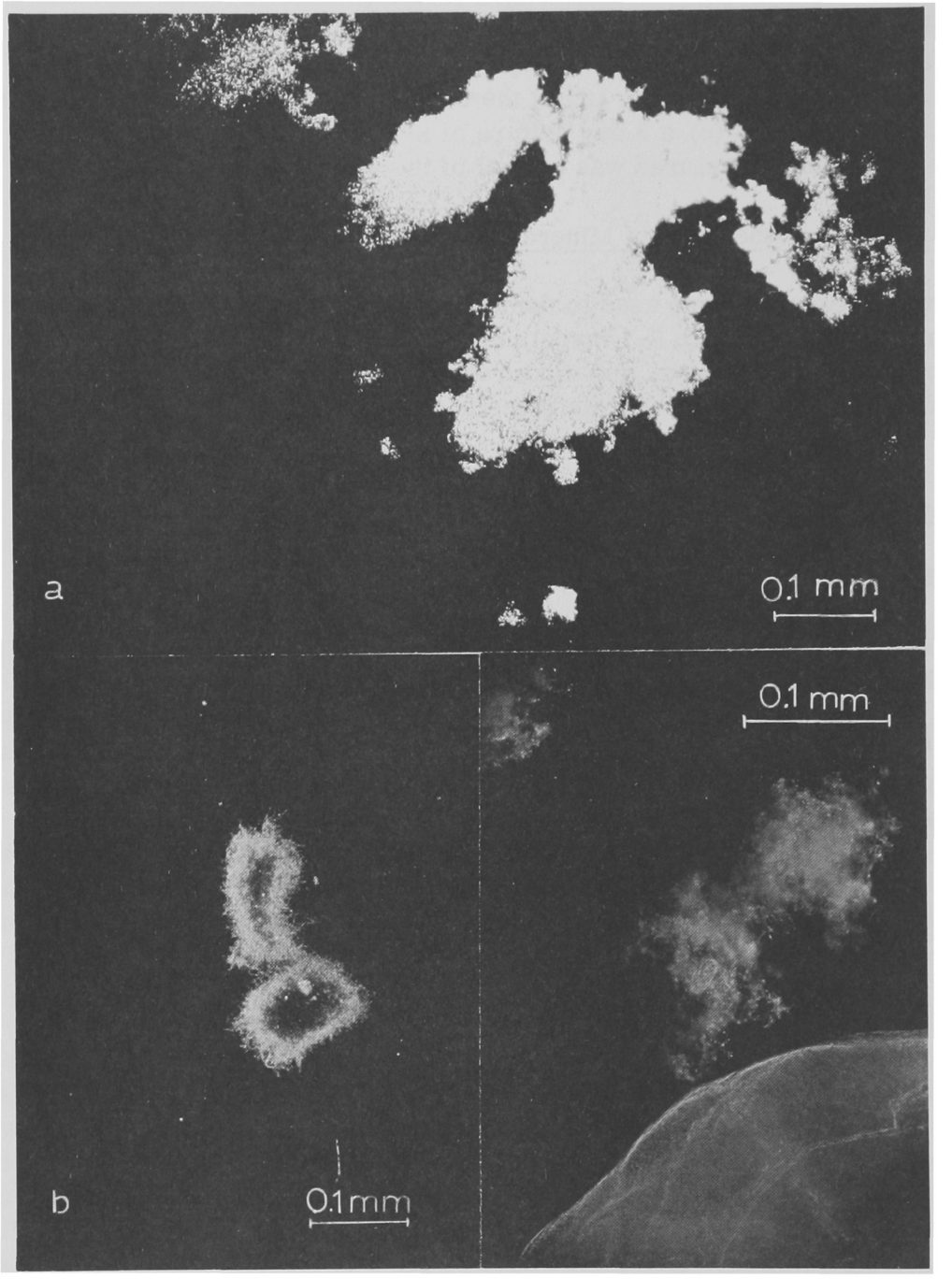

Fig. 15. -- Microscopic appearance of Streptomyces sp. S-1 cultured in media containing chitin or native collagen. (a) Five-day culture of S-1 in minimal salts medium with 0.002M Tris buffer; 0.01M $CaCl_2$ and 0.5 mg/ml of native collagen. (b) Three-day culture of S-1 cells in minimal salts medium with 0.002M Tris buffer, and 0.5 mg/ml of autoclaved collagen. No $CaCl_2$ was added to this medium. About 10% of the mycelial masses had this ringed appearance. See also Fib. 31 for other mycelial clusters from the same culture. (c) Three-day culture of S-1 cells in mineral salts medium with 0.002M Tris buffer and 0.5 mg/ml of autoclaved chitin. The fluffy appearance of colonies viewed with darkfield was typical.

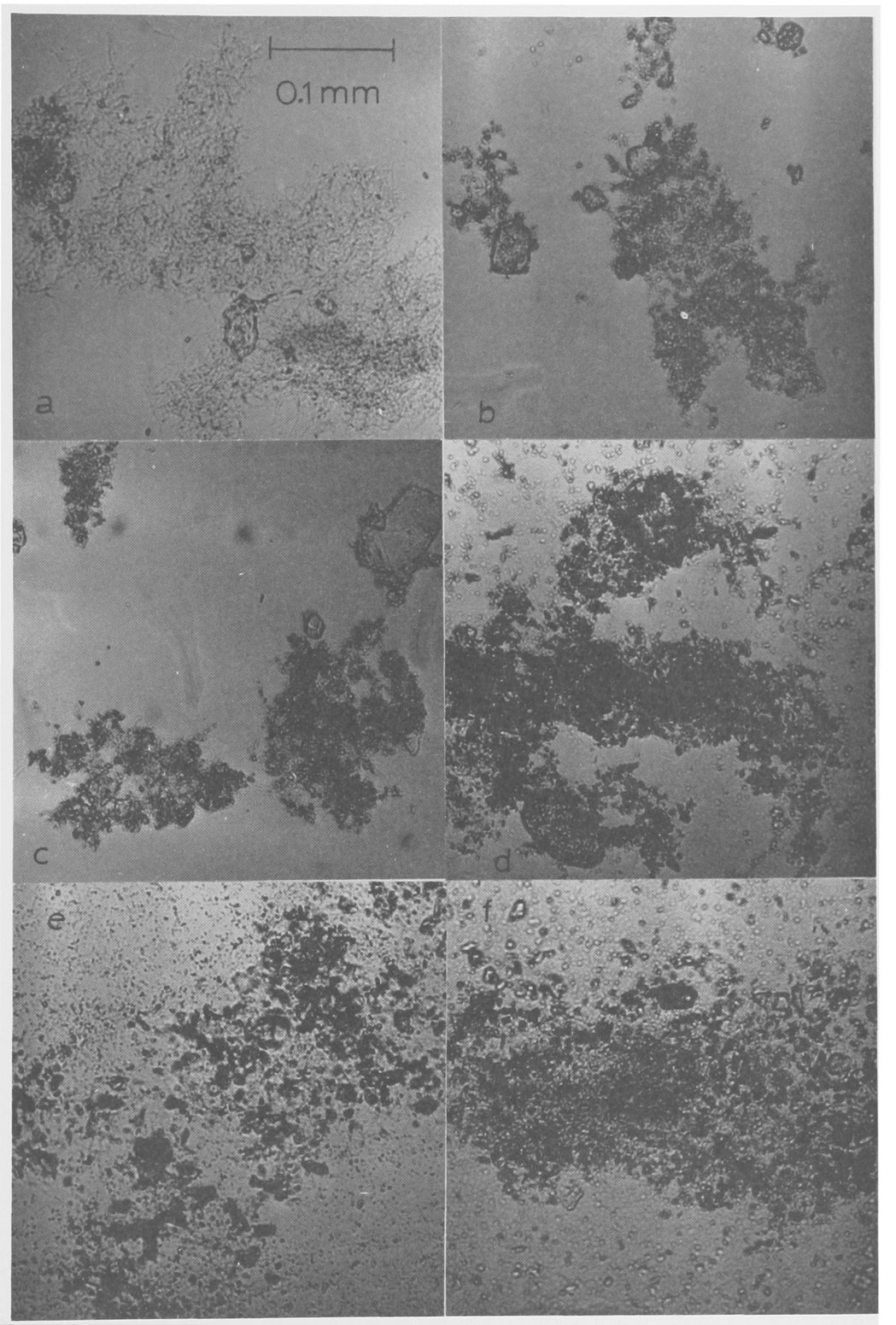

Fig. 16. -- Adherence of kaolinite to Streptomyces sp. S-1 at various pH's. S-1 cells from a 10 day culture in glucose, asparagine, CH_3COONH_4 medium were homogeinzed in the culture medium. (a) control cells, pH 8.2 unadjusted, with no kaolinite added.

(b)-(f): kaolinite added (o.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10. (All photographs are 220X.)

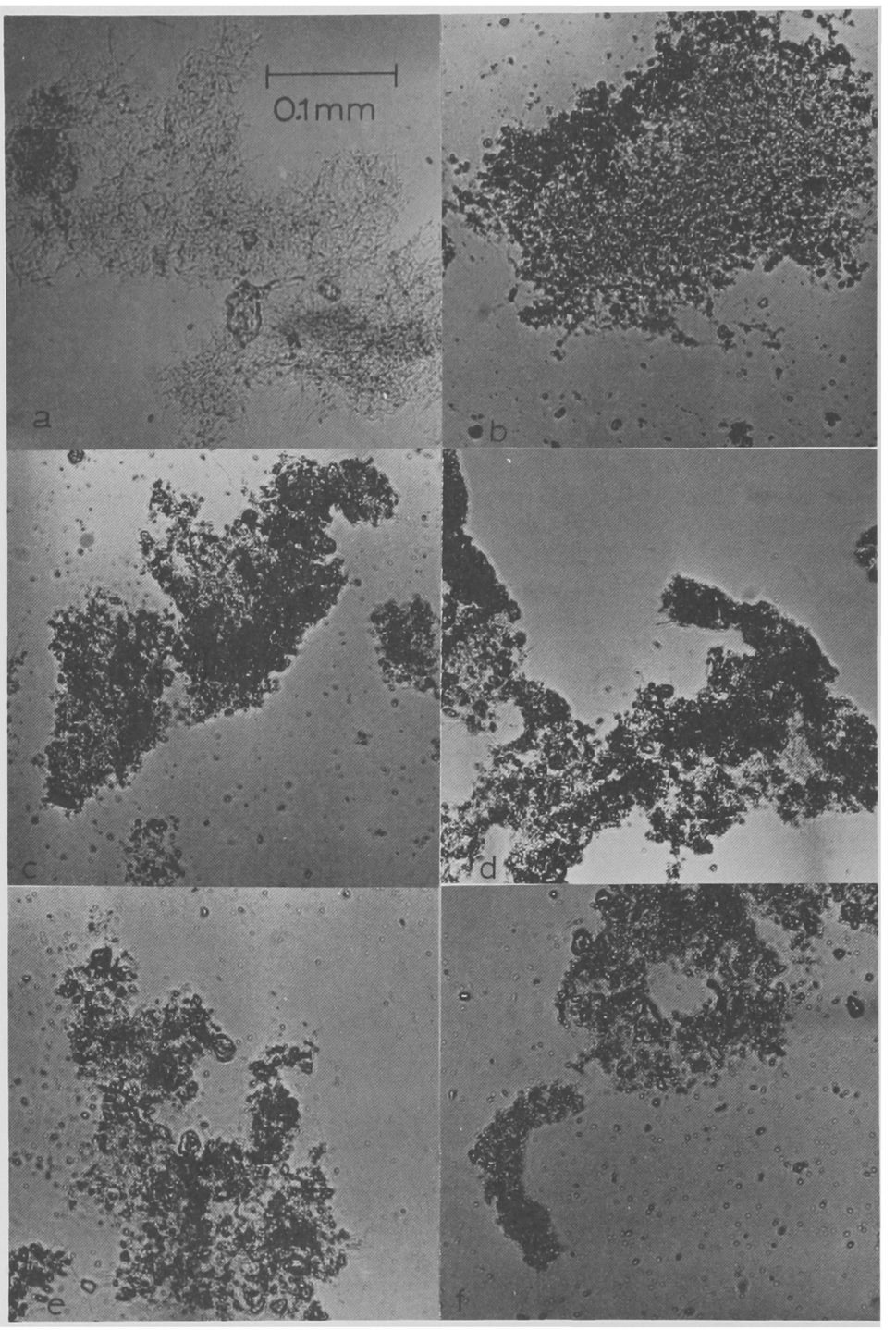

Fig. 17. -- Adherence of illite to Streptomyces sp. S-1 at various pH's. S-1 cells from 10 day culture in glucose, asparagine, CH_3COONH_4 medium were homogenized in the culture medium. (a) control cells, pH 8.2 unadjusted.

(b)-(f): illite added (0.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10). (All photographs are 220X.)

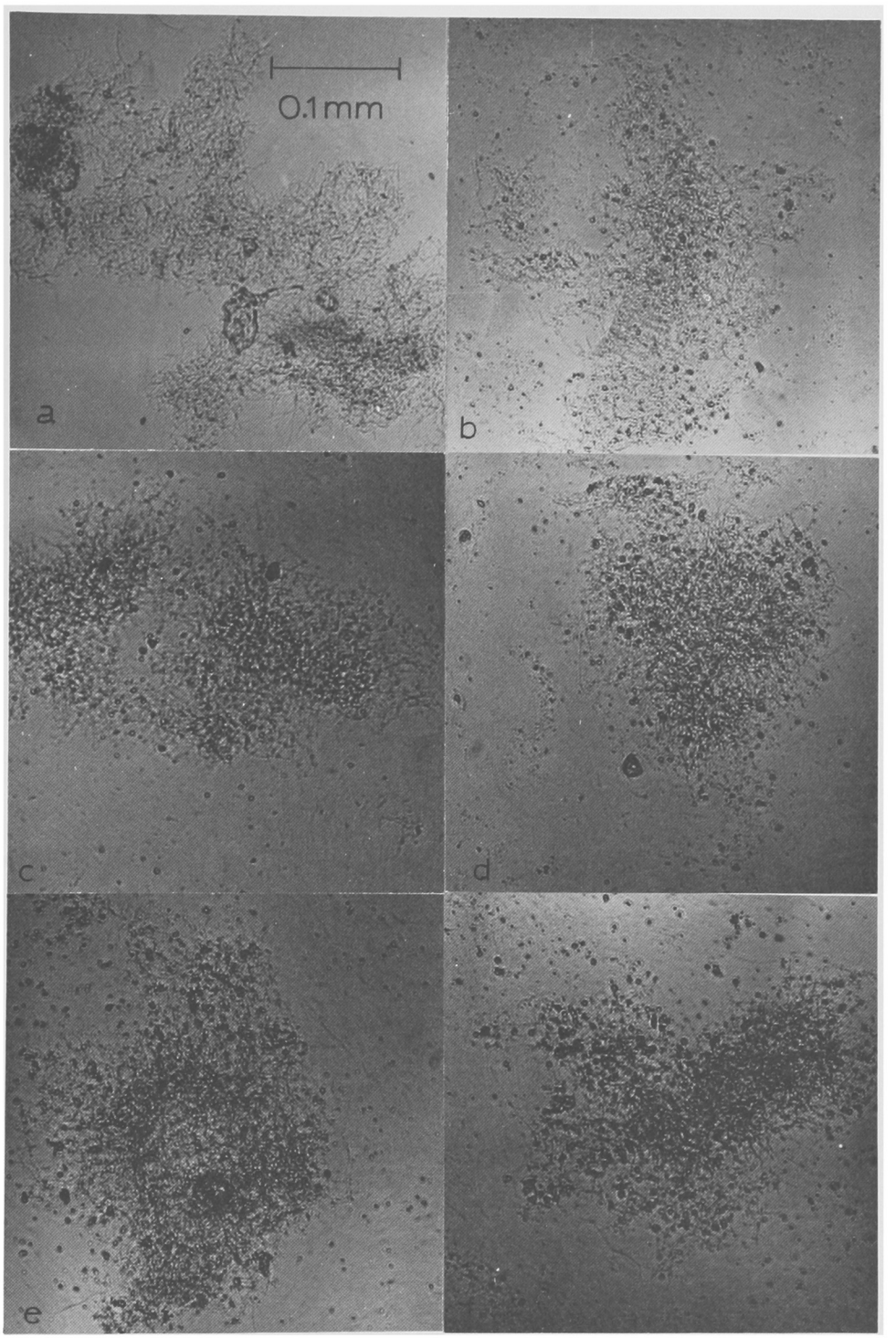

Fig. 18. -- Adherence of bentonite to Streptomyces sp. S-1 at various pH's S-1 cells from 10 day culture in glucose, asparagine, CH_3COONH_4 medium were homogenized in the culture medium. (a) control cells, pH 8.2 unadjusted.

(b)-(f): bentonite added (0.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10. (All photographs are 220X.)

medium demonstrated unequivocally that bentonite, although scarcely visible, was adsorbed to cell surfaces and altered cell density.

Also, due to the small size of particles, bentonite had much higher charge-to-mass ratio. At the arbitrarily chosen particulate concentration, there may well have been more bentonite particles than available adherence sites on the cells.

Effect of time at which mineral was introduced to culture:

Flasks of GM, one containing illite (1 mg/ml) were inoculated with Streptomyces sp. S-1. After 3 days, illite (1 mg/ml) was added to a second culture and mixed briefly before samples were withdrawn from each flask for photography. Fig. 19a shows the characteristic appearance of S-1 colonies incubated with mineral particulate. The illite adhered immediately to the inoculum. Subsequent growth of mycelium produced cell masses in which mycelium + illite formed a central core surrounded by mineral-free mycelium. When illite was added to a 3 day culture of S-1, particles of mineral adhered to peripheral mycelia (Fig. 19b). Because of the increase in cell mass during the 3 day incubation period, illite-to-cell ratio appears to be much lower. In neither case was free illite present in the culture fluid (pH 8.0 - 8.2). All mineral had adhered to mycelial surfaces.

Comparison of Sedimentation Velocities of Cells, Particulates and Mixtures of the Two

Effect of Cell-to-Particulate Ratio at Various pH's at Constant Ionic Strength of Buffer

S-1 cells from a 4 day GM culture were washed once with 0.01M Tris buffer, pH 7.6, and homogenized in fresh buffer. The pH of 5 samples was adjusted to 6.5, 7.0, 7.5, 8.0 and 8.5. To 4 ml cell samples in matched cuvettes, at a constant absorbance at 660 nm of 0.3, 1 ml samples of kaolinite in 0.01M Tris buffer at appropriate concentrations and pH's were added to give final kaolinite concentrations of 0, 0.2, 0.4, 0.5, and 0.8 mg/ml. Absorbance of the samples at 660 nm was read at 0, 10, 20, and 30 min.

The results are presented graphically in Figs. 20-24. The differences in sedimentation velocities of samples at different pH's were so obvious that no statistical analyses were made.

Effect of Cell Age at 5 pH's, at Constant Kaolinite and Cell Concentrations

In several experiments designed to determine optimal pH's and cell-kaolinite ratios for maximum cell-kaolinite aggregation, we noticed that results differed when the cells used had been cultured for different lengths of time. In GM cultures inoculated from YME, S-1 usually began to form large numbers of conidia after 96 hours. To determine whether the age of a culture (and perhaps inception of sporulation) significantly influenced the sedimentation behavior of cell-kaolinite suspensions, we homogenized S-1 cells from 4 day and 5 day GM cultures and diluted them with distilled water to an absorbance of 0.3 at 660 nm. We added 4 ml of kaolinite (1 mg/ml) in Tris buffer (0.01M) at pH's 7.0, 7.5, 8.0, 8.5 and 9.0, to 1 ml samples of the 4 day and 5 day cells. Control tubes contained

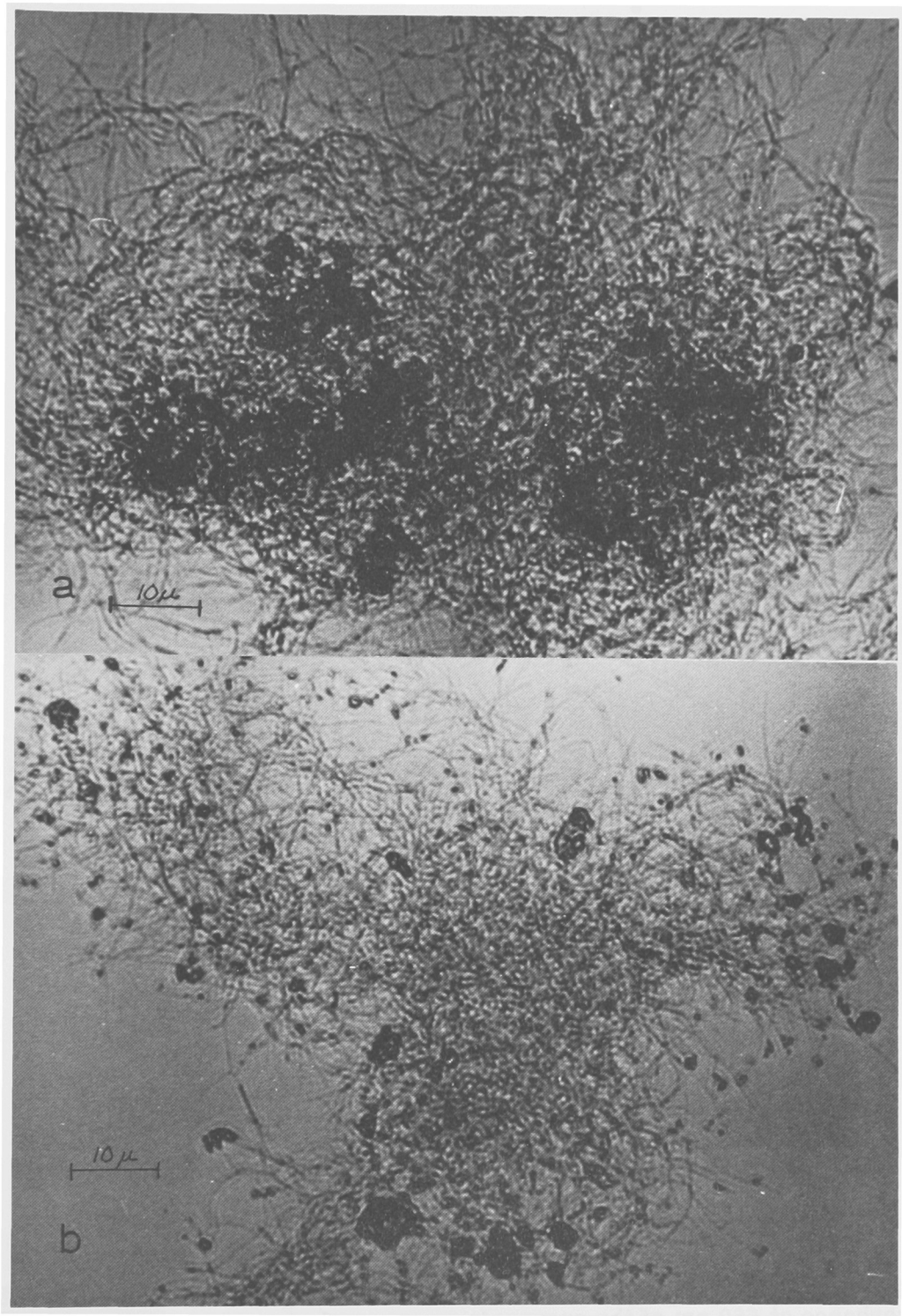

Fig. 19. -- Effect of the time at which illite was introduced in cultures of Streptomyces sp. S-1. One mg/ml of illite was added to duplicate cultures of S-1 in glucose, asparagine, CH_3COONH_4 medium: (a) prior to inocuulation with S-1 cells; (b) after 3 days inoculation of S-1 cells.

Photographs were taken just after illite was added to culture (b). (550X).

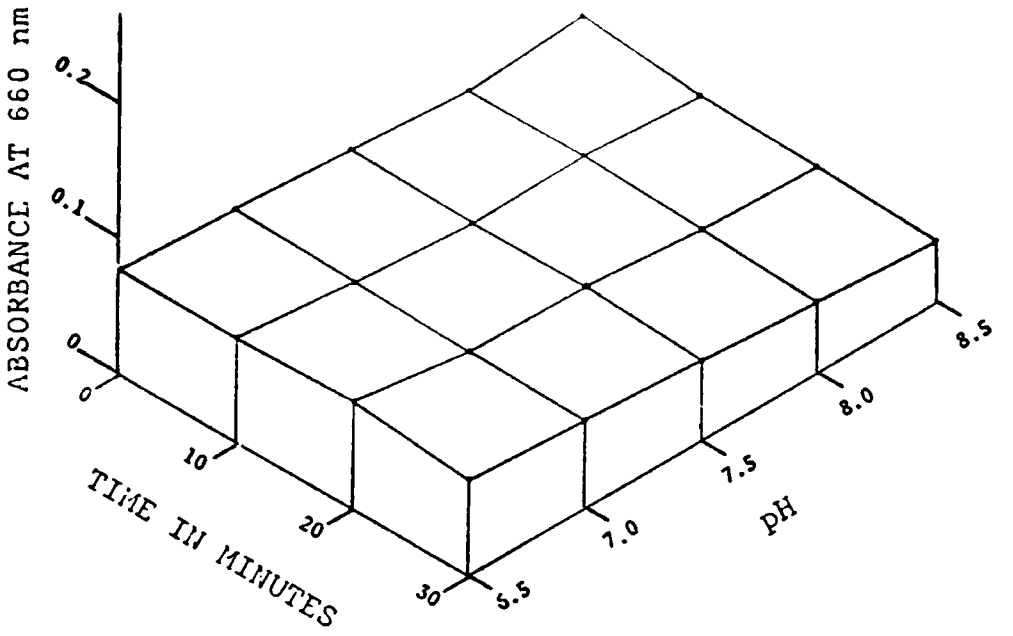

Fig. 20.-- Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance at 660 nm of the suspensions was read at 0, 10, 20, 30 min.

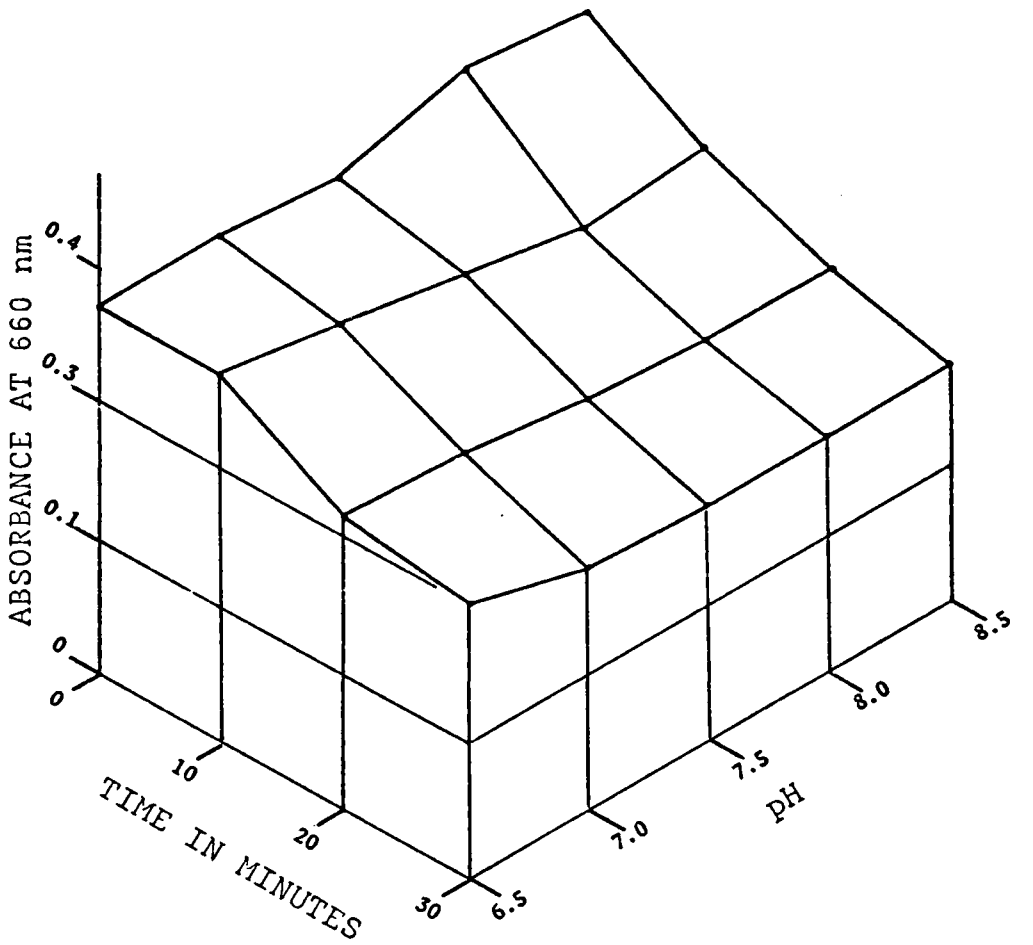

Fig. 21. -- Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (o. 2 mg/ml) at pH 6. 5, 7. 0, 7. 5, 8. 0, and 8. 5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20 and 30 min.

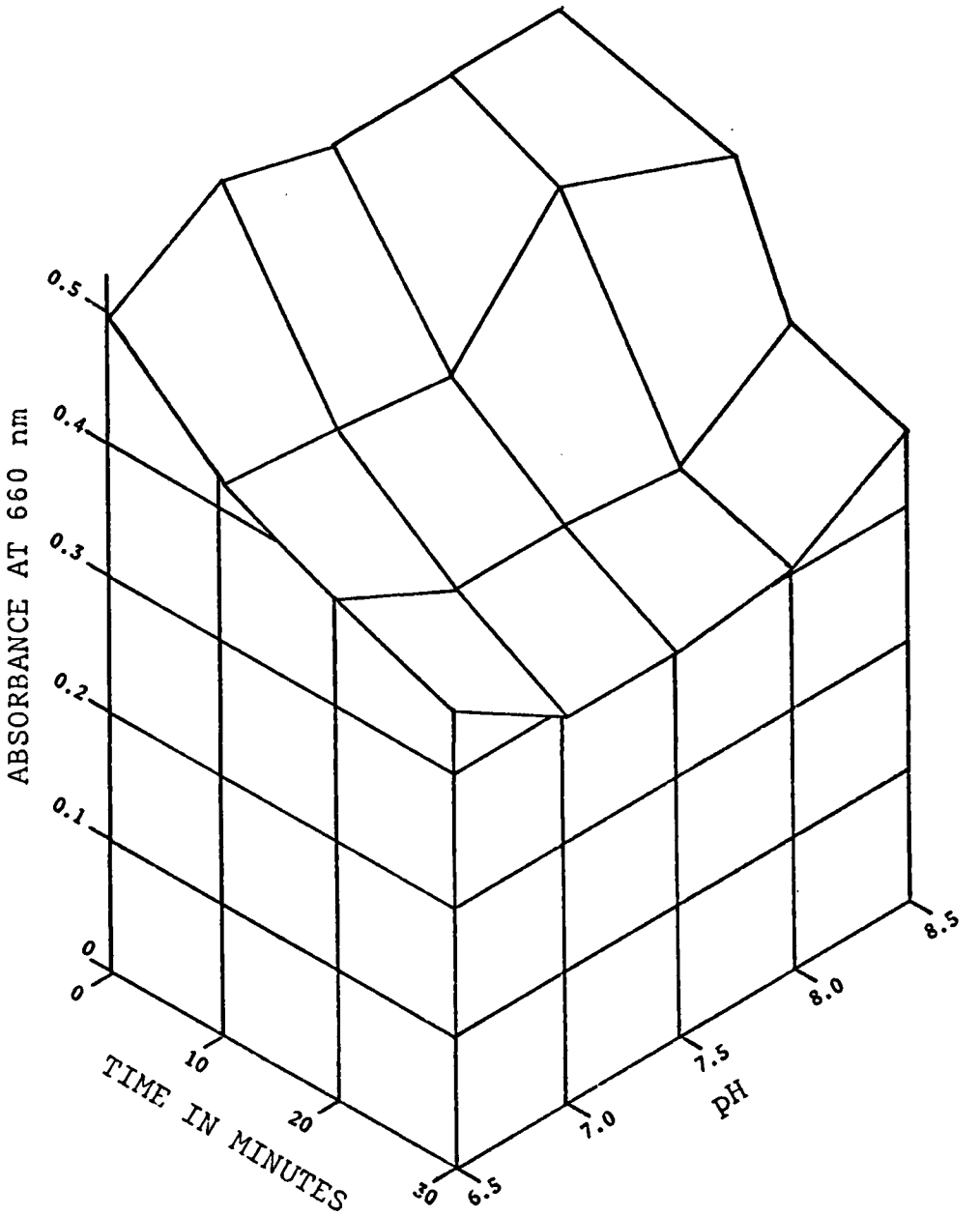

Fig. 22. -- Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.4 mg/ml) at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20, and 30 min.

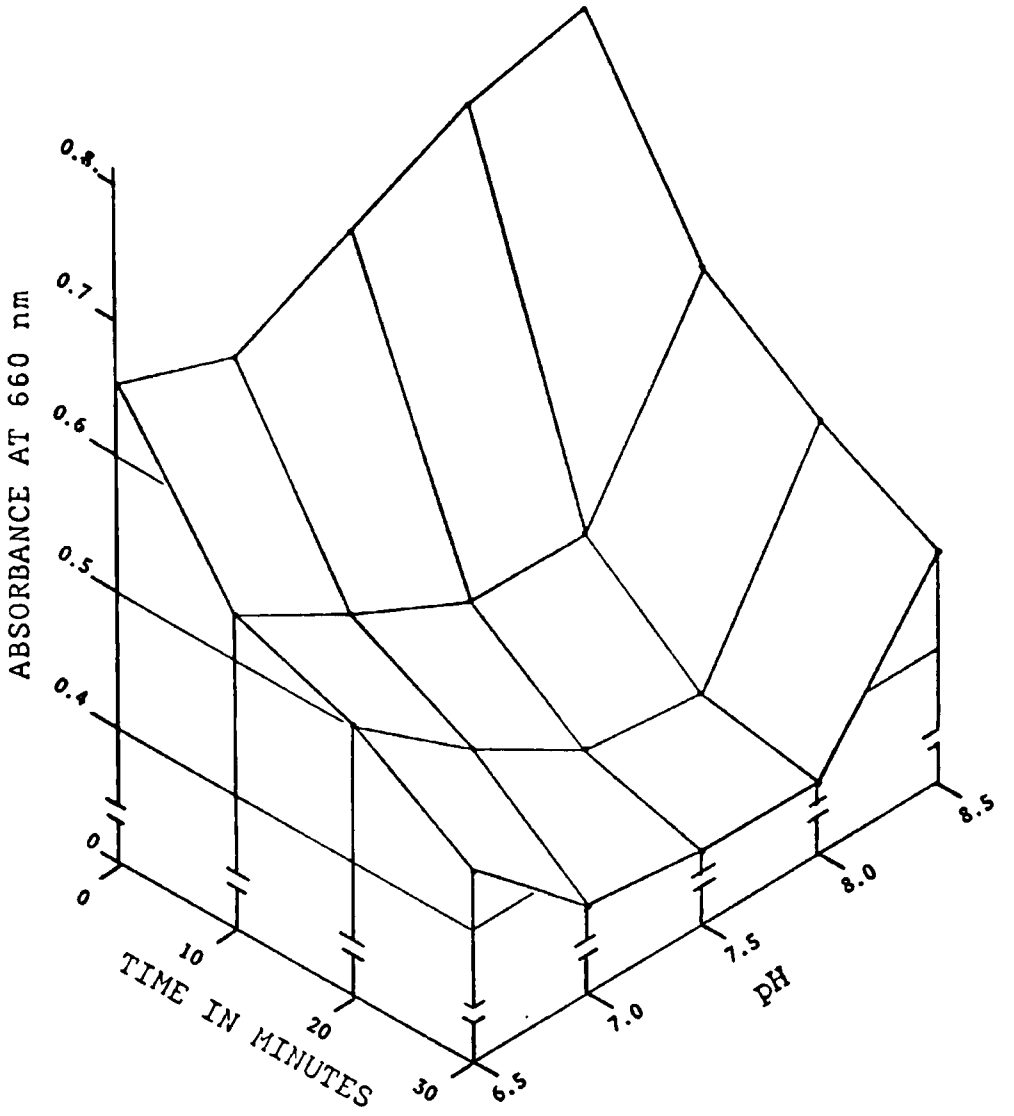

Fig. 23. -- Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.6 mg/ml) at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20, and 30 min.

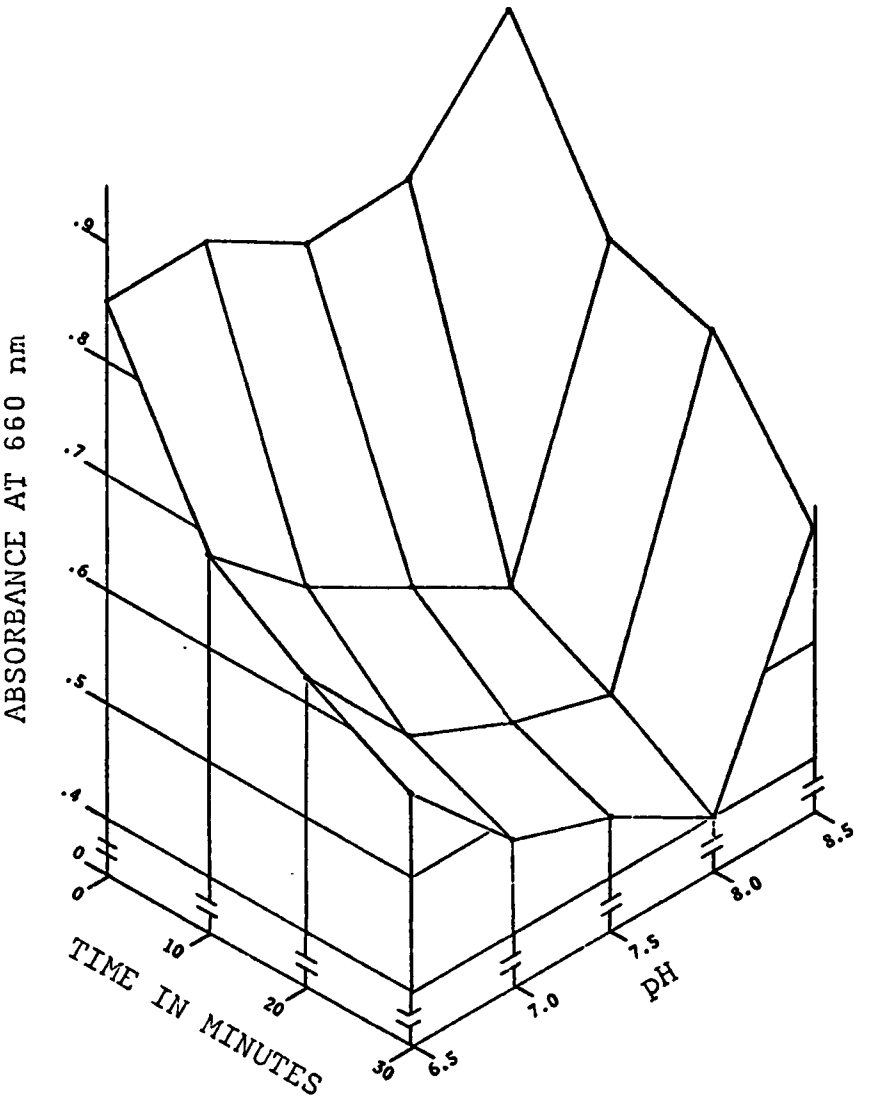

Fig. 24. -- Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.8 mg/ml) at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20, and 30 min.

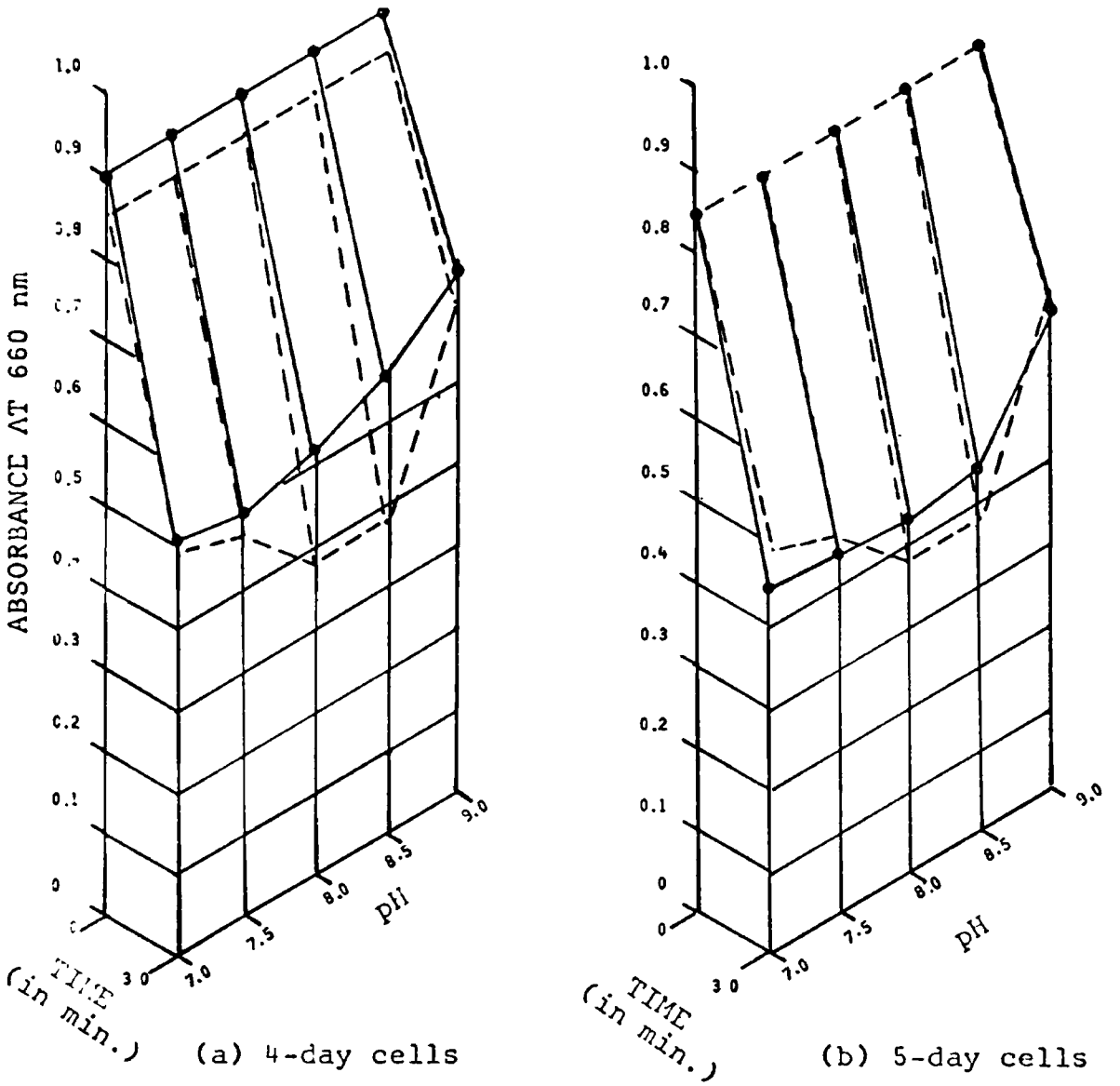

Fig. 25. -- Sedimentation of suspensions of 1 ml of (a) 4-day and (b) 5-day homogenized Streptomyces sp. S-1 added to 4 ml of kaolinite (1 mg/ml) in Tris buffer (0.01M) at pH's 7.0, 7.5, 8.0, 8.5, 9.0. Absorbance of the suspensions at 660 nm was read at 0. a nd 30 min. Cells and kaolinite (—●—); kaolinite(•---).

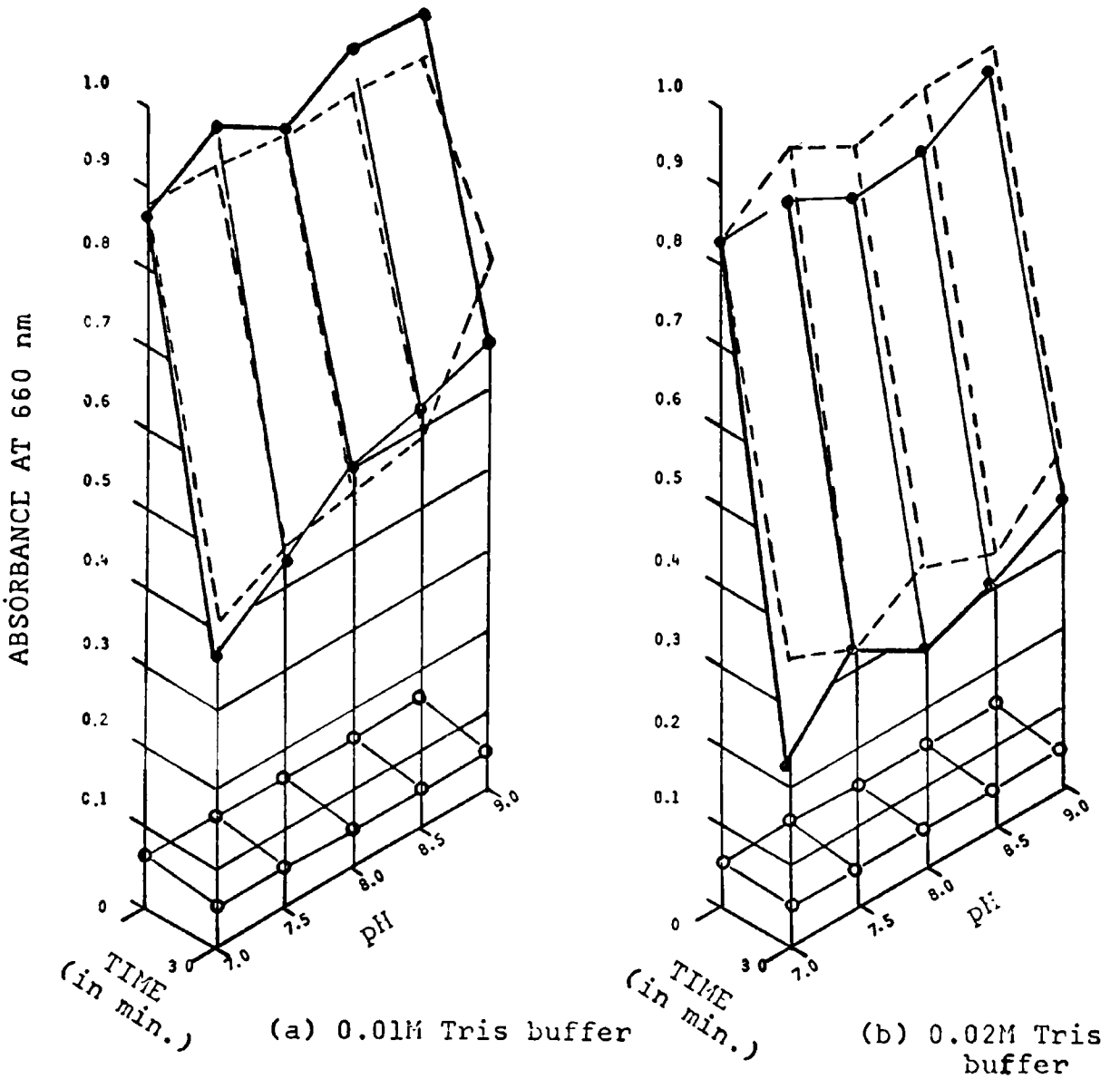

Fig. 26. -- Sedimentation of 1 ml of homogenized Streptomyces sp. S-1 added to 4 ml of kaolinite (1 mg/ml) in (a) 0.01M and (b) 0.02M Tris buffer at pH 7.0, 7.5, 8.0, 8.5 and 9.0. Absorbance at 660 nm was read at 0 and 30 min. Cells only (—o—); kaolinite only (---); cells and kaolinite (—●—).

kaolinite at the various pH's and 1 ml of distilled water. We measured absorbances at 660 nm of triplicate samples of each combination of variables at 0 and 30 min. The difference in absorbance of each sample at the two times was used as an indication of the degree of sedimentation.

The data are graphed in Fig. 15. Sedimentation of aggregates of kaolinite and 4 day cells was greatest at pH 7.5, and decreased as pH increased above 7.5. At all pH's, aggregates of kaolinite and 5 day cells sedimented to a greater extent than kaolinite-4 day cell aggregates, with maximum settling occurring at pH's from 7.0 - 8.5.

A 2-way ANOVA of the decrease in absorbance of the suspensions showed that age was a significant factor (at the 5% level). Differences in pH significantly affected sedimentation of kaolinite and cells of either age (at the 1% level) but the interaction between age and pH was not significant. A 1-way ANOVA of the kaolinite control showed that the pH factor was highly significant at the 1% level.

Effect of Buffer Concentration, at 5 pH's, at a Constant Cell-to-Kaolinite Ratio

To determine whether the concentration of buffer significantly affected aggregation of S-1 cells and kaolinite, we mixed samples of 3 day S-1 cells, homogenized in distilled water (absorbance of cell suspensions: 0.3 at 660 nm), with equal volumes of 0.02M and 0.04M Tris buffers at pH's of 7.0, 7.5, 8.0, 8.5 and 9.0. Samples of kaolinite in Tris buffers at appropriate concentrations and pH's were added to the cell samples so that final concentrations of buffers were 0.01M and 0.02M; of kaolinite, 1 mg/ml. Control samples of cells only, or kaolinite only, at each buffer concentration and pH were included in the test. The absorbance of triplicate samples at each combination of variables was measured at 660 nm at 0 and 30 min. The data are presented graphically in Fig. 26. Each point plotted is the average of 3 determinations. The differences in absorbance of tubes at 0 and 30 min. were analyzed by 2-way ANOVA. Table 6 lists the significance of buffer concentrations, pH and the interaction of the 2 factors in the control and cell-kaolinite samples. The pH, buffer concentration, and their interaction all significantly influenced the rate at which kaolinite sedimented. Of the three effects, buffer concentration had the most pronounced influence on kaolinite sedimentation.

Kaolinite was the component of the cell-kaolinite samples which was influenced by buffer concentration and pH. Neither buffer concentration nor pH significantly influenced sedimentation of S-1 cells in the absence of kaolinite, but in the mixture of cells and mineral, both buffer concentration and pH significantly influenced the decrease in absorbance of the suspensions.

Density Gradient Centrifugation

To demonstrate that the stability of the adherence of S-1 mycelium to mineral particulates, we layered cell-mineral aggregates on linear density gradients of Renografin and water (density range 1.0 - 1.46). The cells, if not attached to particulates, would remain in the upper portion of the gradient in the range of 1.12. The particulates, however, having a mean density of 2.7, would be expected to pellet below the pure Renografin. Cells to

TABLE 6

Significance of buffer concentration, pH, and their interaction in the sedimentation of S-1 cells and kaolinite.

Two-way analysis of variance.

Sample	Effect	Significance[a]
Kaolin only	buffer concentration	**
	pH	*
	interaction	*
Kaolin + cells	buffer concentration	**
	pH	*
	interaction	NS
Cells only	buffer concentration	NS
	pH	NS
	interaction	NS

[a] * : significant at the 5% level
**: significant at the 1% level
NS: effect not significant

which no kaolinite was added, did indeed remain in the upper 2 ml of the gradient, but clumps of cell-mineral aggregates were distributed throughout the gradient tubes. Very little mineral pelleted. Fig. 27 is an interesting comparison among cells which had been incubated with the mineral in the medium; those to which particulate had been added after incubation, just prior to centrifugation; and cells to which no particulate had been added. Cells incubated with kaolinite or bentonite formed bands at a lower level in the tube (hence a higher density) than those to which kaolinite had been added just before centrifugation. The gradient tubes were fractionated and photographs made of cell clusters removed from the various fractions. Cells incubated with mineral had adhered to the mineral and formed clusters of cell and mineral which formed a core surrounded by subsequent growth. The cell clusters were large and dense. In the sample to which kaolinite had been added just prior to centrifugation, the mineral was found adhering to the periphery of cell clusters in loose array. Micrographs of cell-mineral aggregates removed from different fractions show that as the density of the fraction increased, the kaolinite-to-cell ratio also increased (Fig. 28). Even at the forces exerted on the aggregates by the centrifugation through a gradient, there was virtually no free mineral at the bottom of the tubes and no free cells at the top of the gradient. Fig. 27 is a print made from a reversal slide; the dark areas appearing in the picture at the bottoms of the tubes are due to reflected light.

Centrifugation on a density gradient was also used to try to separate kaolinite from HPA. A linear Renografin gradient was layered with various kaolinite-to-HPA ratios. The results were the same as those for kaolinite-cell aggregates. HPA particles to which more kaolinite adhered, were lower in the gradient. Collagen, with a density of about 1.12 would not have penetrated the dense Renografin solution to lodge at a level where the density exceeded 1.45, had not the kaolinite remained attached to increase the density of the aggregate. Since the force of this centrifugation was 13,200 x g., it is obvious that kaolinite is not merely resting on the surface of the HPA particle in Fig. 29.

Adherence of S-1 Mycelium to Substrates

In considering the hypothesis that microbial substrate utilization is enhanced by intimate contact among organism, enzymes and substrates, it is important to know whether Streptomyces does adhere to particulate substrates. Into MSM-2TB containing collagen, MSM-2TB containing chitin, and MSM-SO_4-2TB containing cellulose, we inoculated S-1.

Collagen:

Fig. 30 is a micrograph of S-1 mycelia surrounding particles of collagen. In a 3 day culture, not every mass of mycelium enveloped a mass of collagen, but every collagen particle examined had mycelium adhering to it.

Chitin:

Particles of chitin appeared macroscopically to remain free in the medium of a 3 day culture, but microscopic examination showed that colonies of S-1 adhered to edges and surfaces (Fig. 31). Some small flakes of chitin were embedded in masses of mycelium.

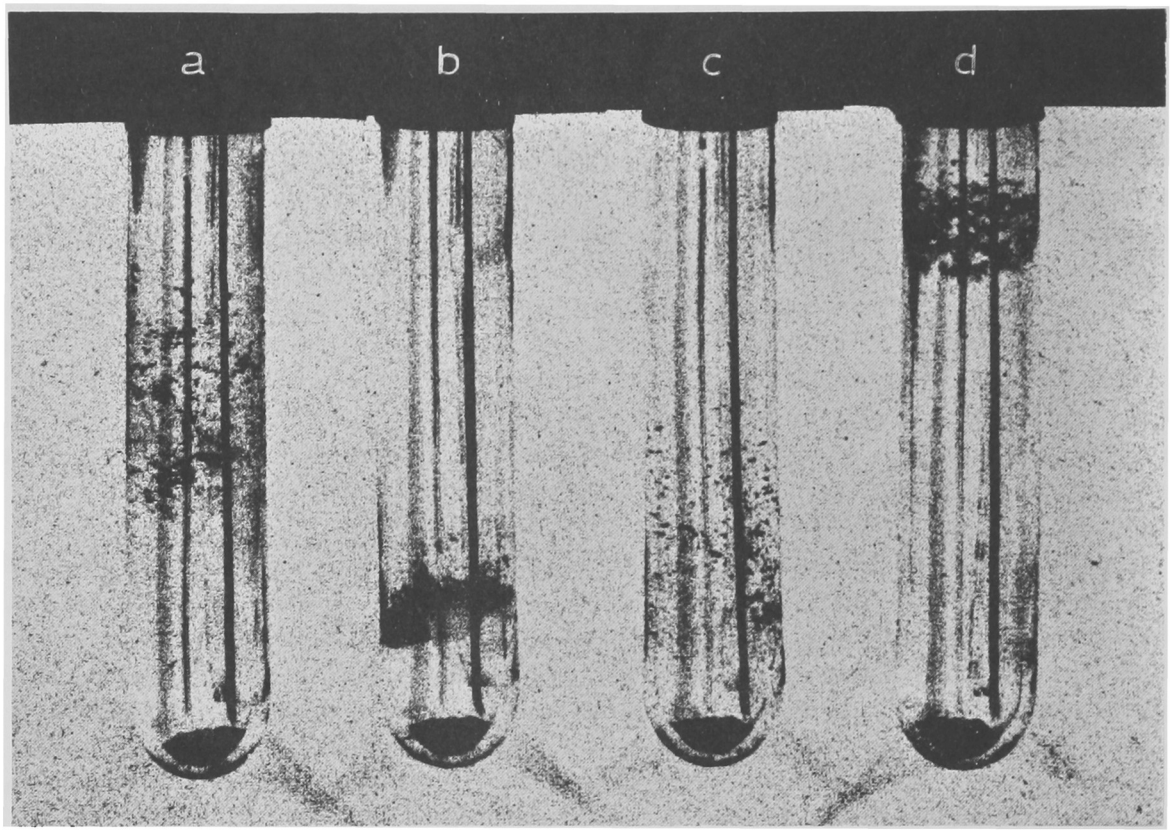

Fig. 27. -- Formation of cell-mineral aggregates of Streptomyces sp. S-1, demonstrated by density gradient centrifugation on a linear water: Renografin gradient. The 4 gradient tubes are pictured: (a) Sample of S-1 culture to which kaolinite was added just prior to formation of the gradient; cell-mineral aggregates were dispersed through a wide density range. (b) Sample of S-1 cells cultured with bentonite; cell-mineral aggregates formed a sharp band, with high density. (c) Sample of S-1 cells cultured with kaolinite; cell-mineral aggregates formed a diffused band in an area of greater mean density than the band in tube (a). (d) Sample of S-1 culture to which no mineral was added; cells remained near the top of the tube where the density of the suspending fluid was lowest. Mineral concentration was 0.5 mg/ml of culture medium. No pellets formed in the tubes. The photographs were printed from a reversal film. The dark areas at the bottoms of the tubes are due to reflected light.

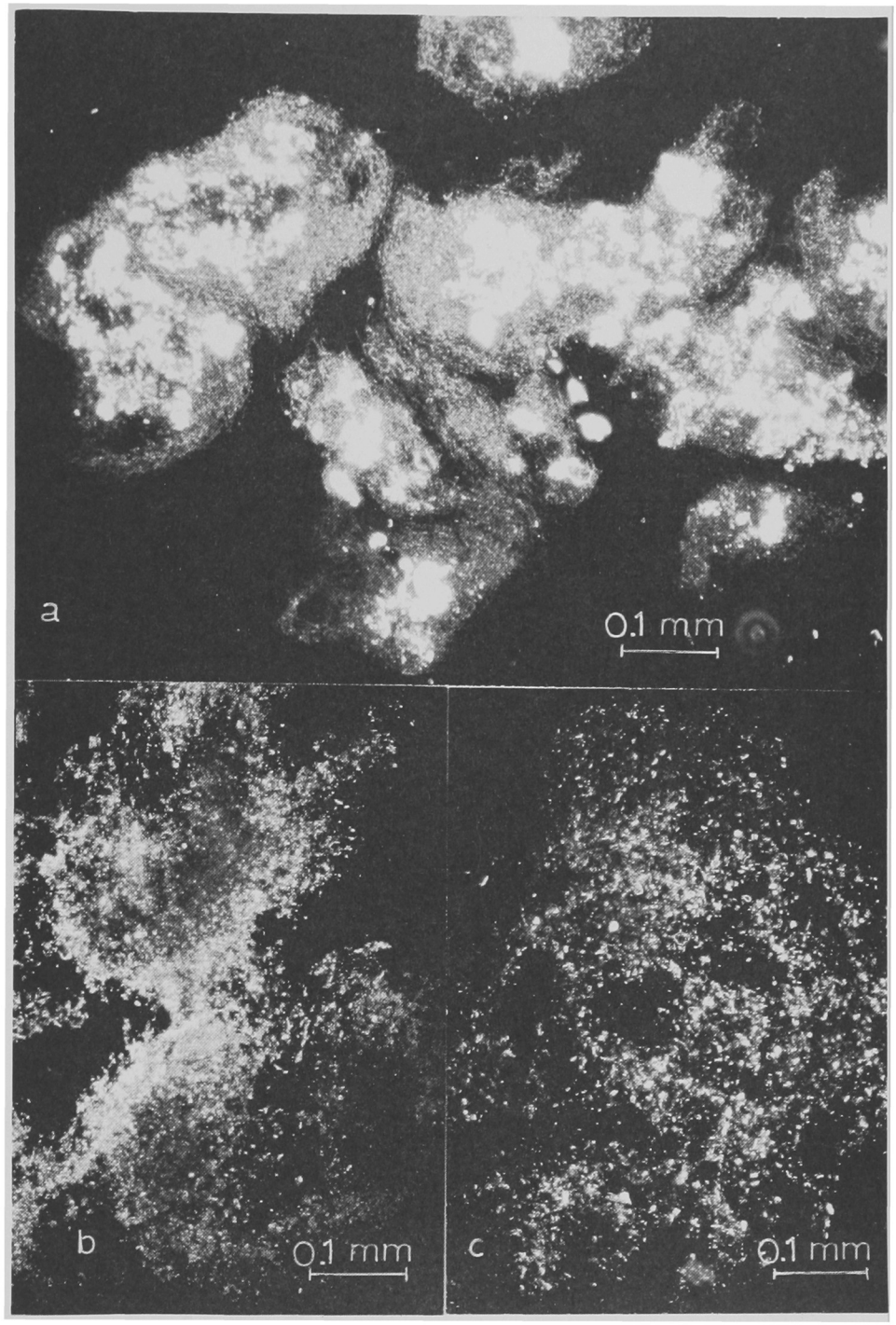

Fig. 28. -- Adherence of kaolinite to Streptomyces sp. S-1 demonstrated by density gradient centrifugation. (a) cells were cultured in chemically defined medium containing 0.5 mg/ml kaolinite; (b) and (c) 1.0 mg/ml of kaolinite was added to control culture after incubation. Samples were layered on linear water: Renografin gradients and centrifuged at 13,200 x g for 1 hr. Densities of fractions of the gradients were determined by measuring the refractive index of the suspending fluid. Densities of the aggregates pictured were: (a) 1.24; (b) 1.23; (c) 1.38. The density of cells was about 1.12; of kaolinite, about 2.7.

Fig. 29. -- Adherence of kaolinite to Hide Powder Azure demonstrated by density gradient centrifugation. Aggregates of kaolinite and Hide Powder Azyre (HPA) were layered on linear water:Renografin gradients. The density of HPA is about 1.12; of the aggregate shown was about 1.45. Crystals of kaolinite can be seen adhering to the HPA particles (arrows).

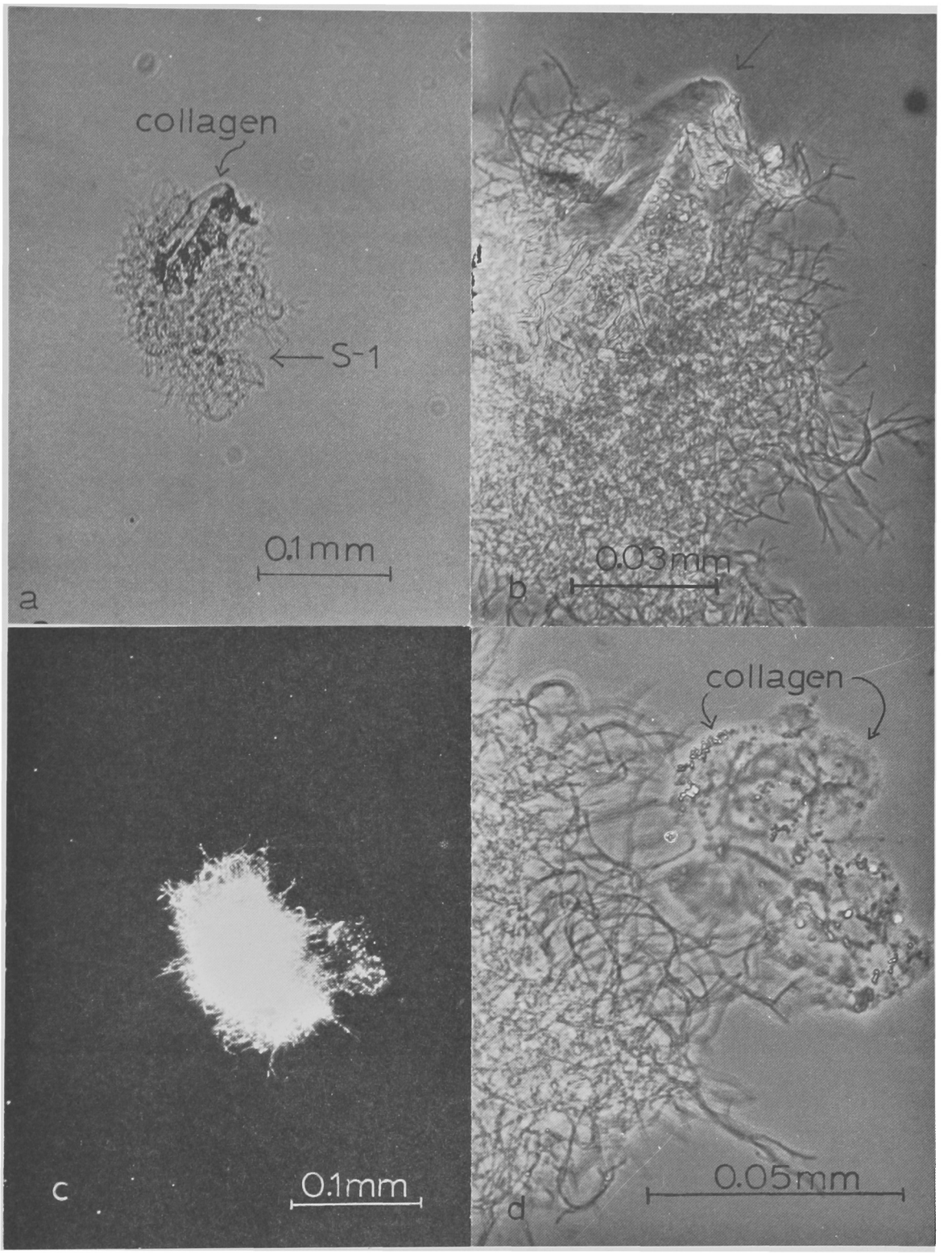

Fig. 30. -- Adherence of Streptomyces sp. S-1 to a particulate substrate: collagen. Cells were cultured for 3 days in minimal salts medium with 0.002M Tris buffer and 0.5 mg/ml of autoclaved collagen. (a) and (b) are two views of the same myeelial cluster surrounding a collagen fiber (arrow). (c) and (d) are two views of another mycelial mass adhering to a piece of collagen (arrow).

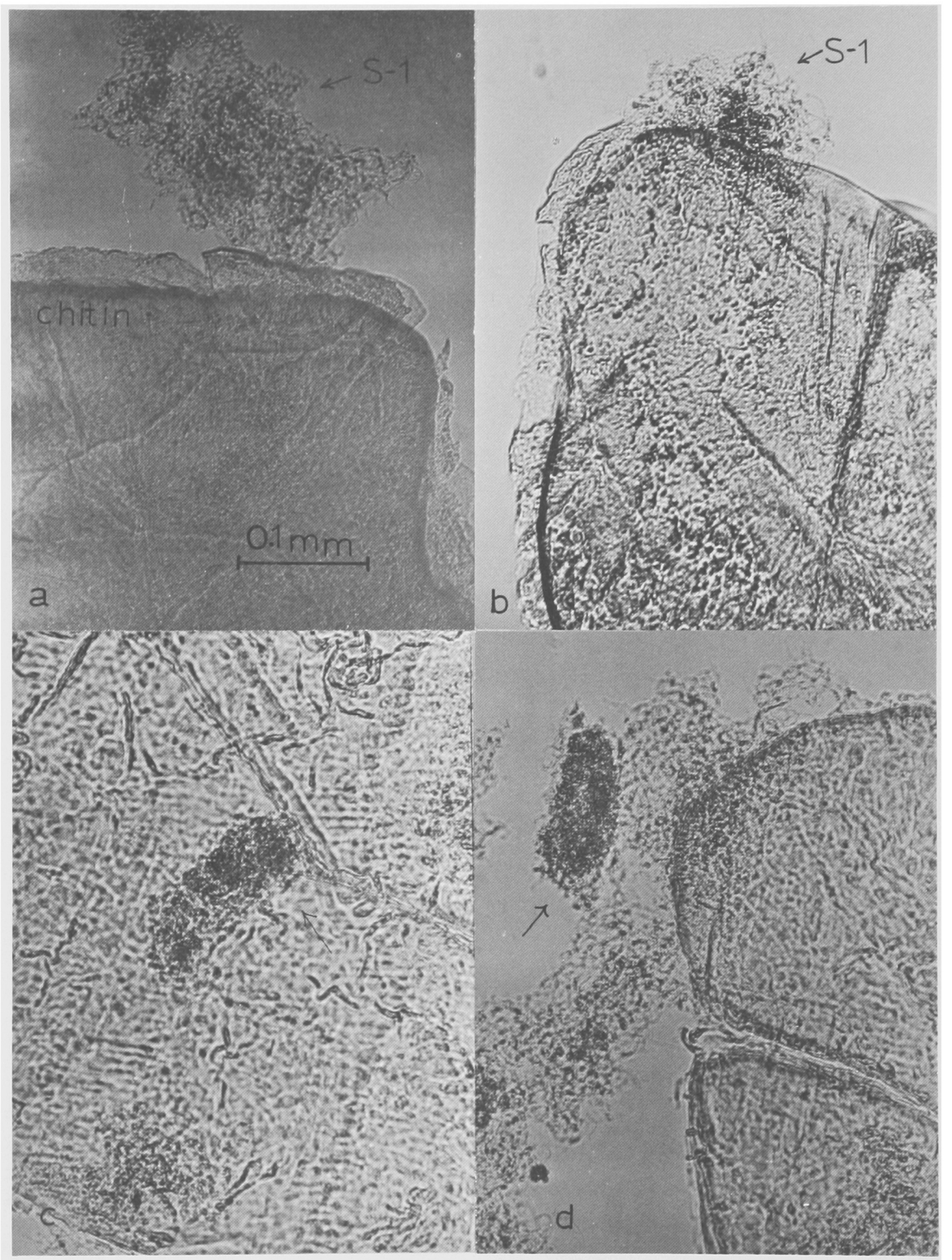

Fig. 31. -- Adherence of Streptomyces sp. S-1 to a particulate substrate: chitin. Cells were cultured for 3 days in minimal salts of chitin. Colonies can be seen adhering to the edges of a chitin flake surfaces (c) and (d) (arrows).

Cellulose:

By the third day, little free cellulose remained in the culture fluid. Mats of cells and cellulose encircled the "high water line" of the shaker flask. Fig. 32 shows a typical view of aggregates, stained with crystal violet, from a 14 day culture. Networks of mycelium enmeshed cellulose fibers, in many cases, binding several together.

Adherence of Active Enzyme to Mineral Particulates

Mineral unwashed after exposure to enzyme:

The procedure described for testing the activity of M. chalcea protease was used to test the proteolytic proficiency of S-1. CMF from a 4 day culture of S-1 was added to sterile kaolinite to give a final concentration of 0.4 mg/ml. The low concentration of kaolinite was chosen to increase the probability that all possible kaolinite surfaces would contact enzyme molecules. The kaolinite was removed by centrifugation and added to a sterile suspension of HPA (0.25 mg/ml) in 1 ml of Tris buffer at pH 7.6. The suspension was shaken and kaolinite-HPA aggregates allowed to settle. These were removed by Pasteur pipette and added to 5 ml of 1% agar at 64 C. One HPA particle (0.25 mm x 0.65 mm) was photographed at intervals for 135 min. Fig. 33 is a series of these pictures taken at 12, 32, 62, 90, 105, 120 and 135 min. (A similar preparation from CMF to M. chalcea required more than 48 hrs to accomplish the same amount of degradation and dye release.) Not only dye release was seen by the microscopist and recorded by the camera. Loss of structure occurred at a rate which was readily recorded by 30 min. photographic intervals, and was visible to an observer during the latter half of the dissolution process. As we watched the HPA particle, strands of fiber seemed to break loose - sometimes with a snapping motion - and float in what appeared to be a liquid matrix within the area originally occupied by the collagen particle. Structural disintegration preceded dye release in some areas of the particle. A careful scrutiny of the photographs of Fig. 33 will reveal structures which have changed orientation between photographic exposures. Dye release can also be detected in the pictures not only by loss of density in the particle area, but by the increased density of the surrounding area. The mottled appearance of the background area of agar is due to diffusion of dye. One detail which can be seen very well in color films is the adherence of dye to the kaolinite which originally clugn to the collagen particle. As the HPA particle decolorized, the surrounding kaolinite became progressively more blue.

Minerals washed after exposure to enzyme solution:

Pooled culture media from 4 day and 11 day GM cultures of S-1 were filtered to remove cells and spores. Sterile particulates (0.5 mg/ml) were added to samples of the CMF. One CMF sample was retained as a control, but was centrifuged with the other preparations each time. Samples were centrifuged at 40,000 x g for 1 hour. A sample of each supermatant fluid and the control was removed for protein estimation. The rest was decanted to another sterile centrifuge tube and another 0.5 mg/ml sample of the same particulate was added to the three supernatant fluids (designated KS_1, IS_1, BS_1) which had contained particulate. These were centrifuged again at the same force, for the same time. The mineral pellets (designated K_1, B_1, and I_1) removed by the first centrifugation, were

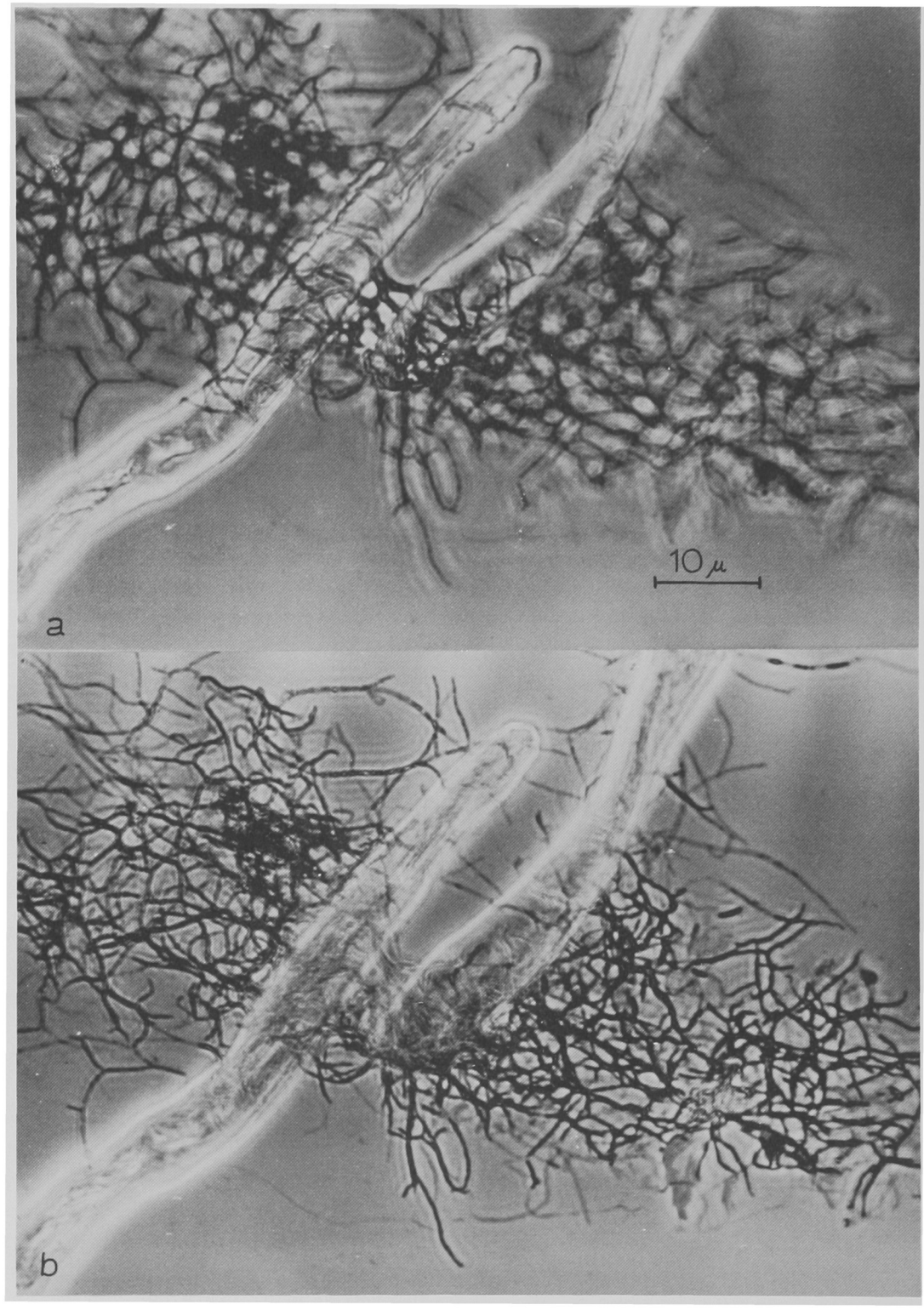

Fig. 32. -- Adherence of Streptomyces sp. S-1 to a particulate substrate: cellulose. Cells were cultured for 9 days in minimal salts medium with 0.002M Tris buffer, 1% (w/v) CH_3COONH_4, and (b) are two views, at different focal levels, of cells and cellulose stained with crystal violet. Strands of mycelium are wrapped around cellulose fibers and are bridging the area between 2 fibers.

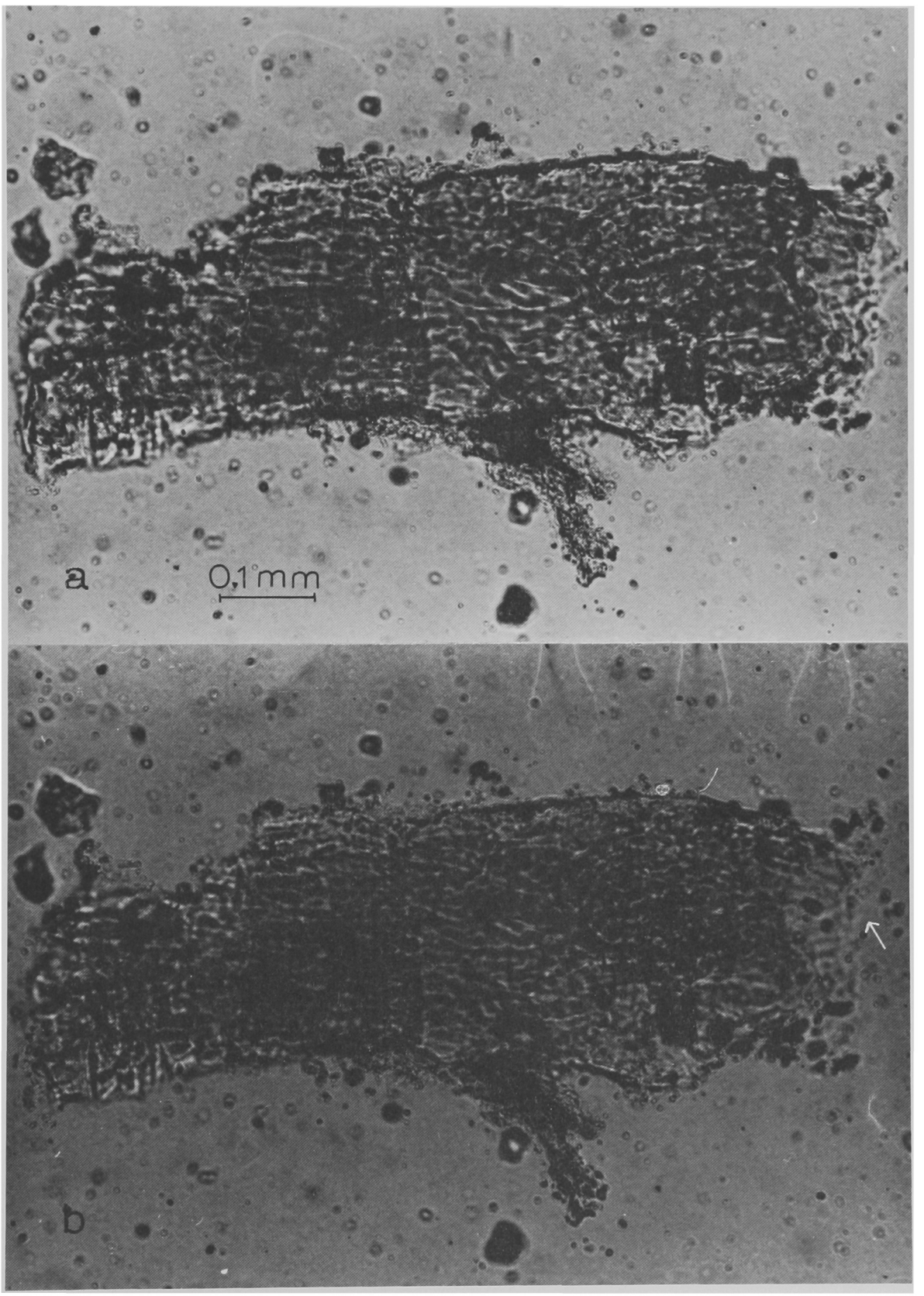

Fig. 33. (a) (b)

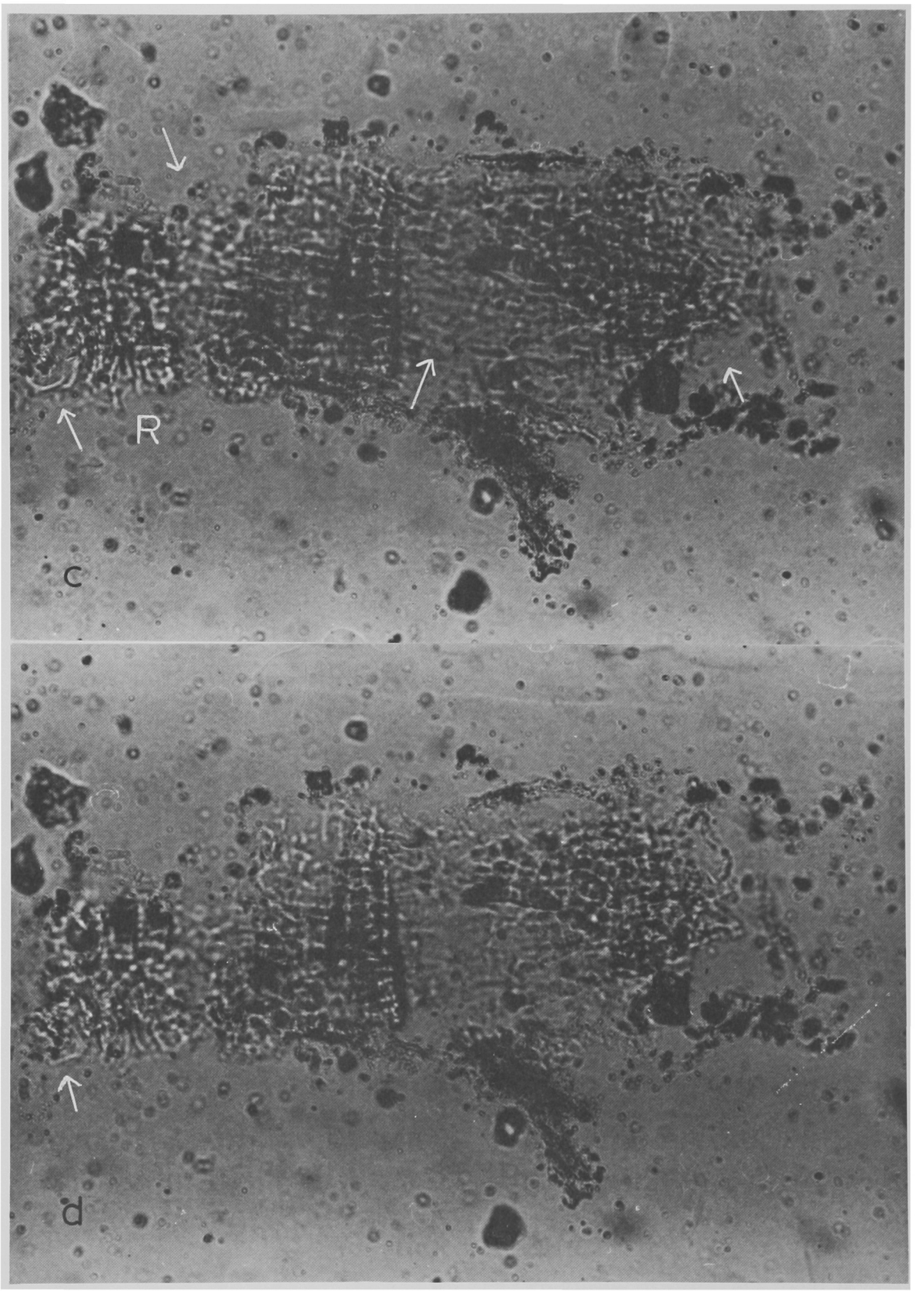

Fig. 33. (c) (d)

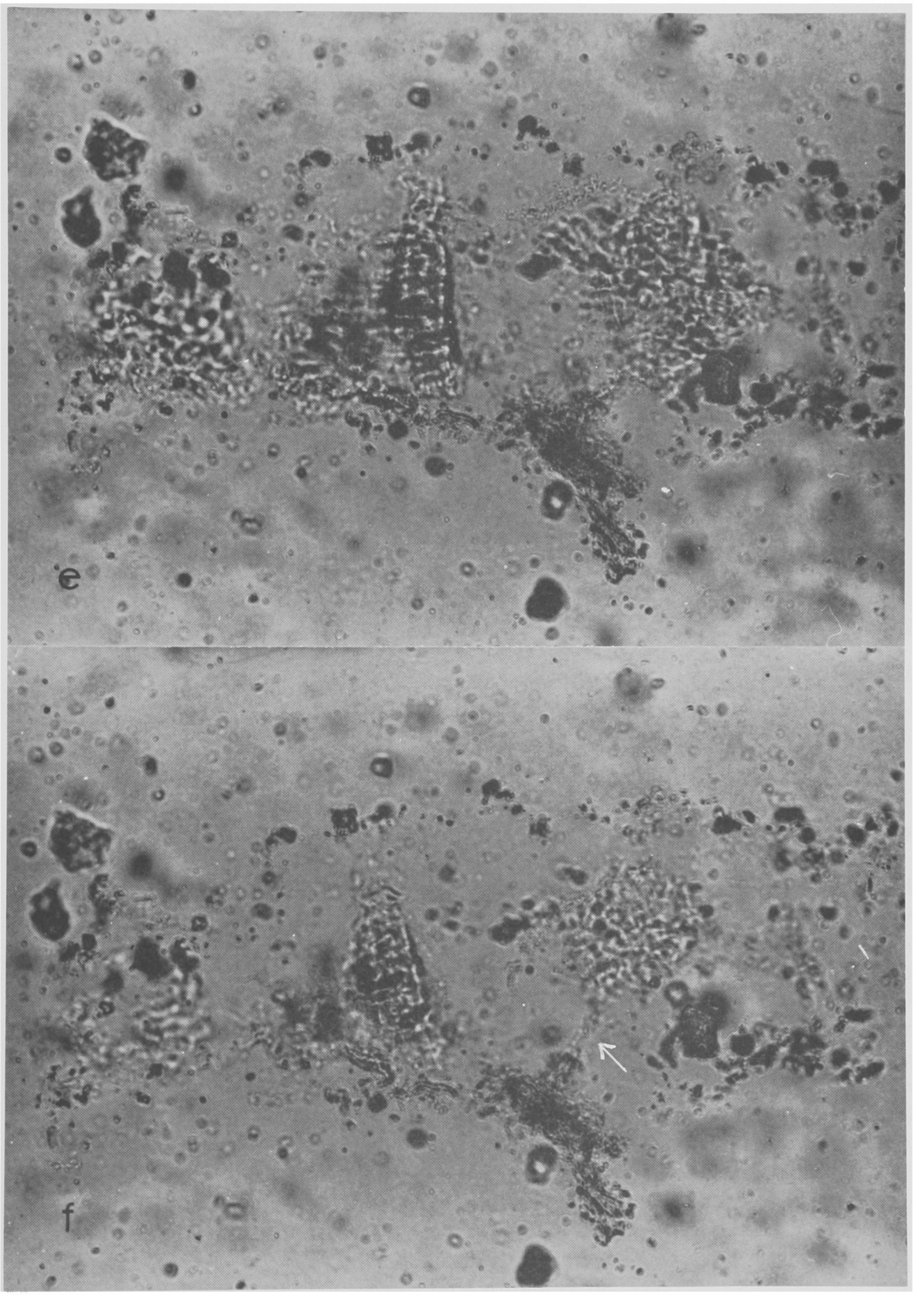

Fig. 33. (e) (f)

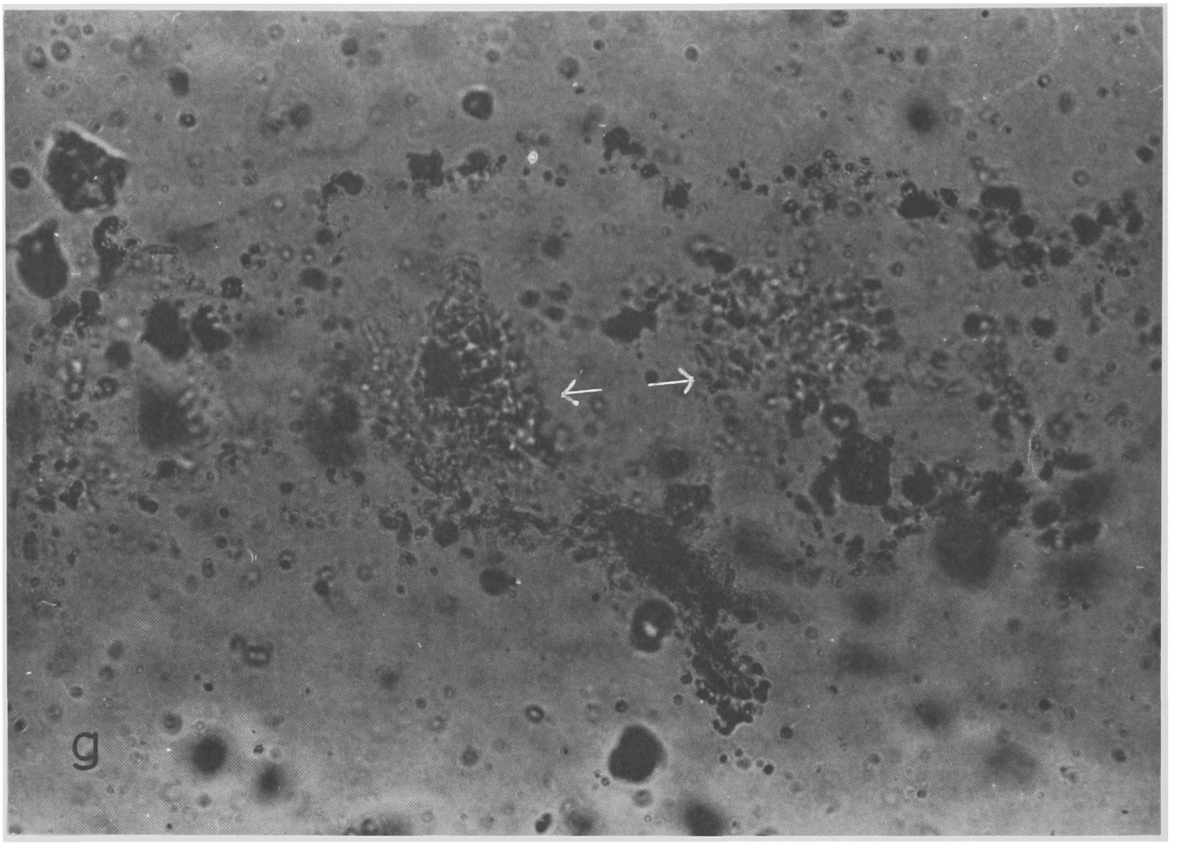

Fig. 33. (g)

Fig. 33. -- Dissolution of Hide Powder Azure (HPA) by Streptomyces sp. S-1 enzyme absorbed to kaolinite. Kaolinite at 0.4 mg/ml was added to a cell free filtrate of glucose, asparagine, CH_3COONH_4 medium in which the streptomycete had been cultured for 4 days. The kaolinite was removed by centrifugation and added to 1 ml of 0.01M Tris buffer, pH 7.6 containing 0.25 mg of HPA. The suspension was shaken and kaolinite-dye aggregates allowed to settle. These were removed by Pasteur pipette and added to 5 ml of 1% agar at 46 C. One HPA particle 0.25 mm x 0.65 mm was photographed at intervals for 135 min. Times given are the number of minutes after first contact of HPA with kaolinite-adsorbed enzyme preparation: (a) 12 min; (b)32 min. The arrow indicates an area where loss of structure has begun to occur. (c) 62 min. Dissolution of fiber is evident in several locations (arrows). Strands have reoriented at R. (d) 90 min. Strands in the area of the arrow could be seen moving in an apparently liquid matrix. (e) 105 min. The mottled appearance of the background agar is due to diffusion of dye from the site of the HPA particle. Movement within the particle was visible. (f) 120 min. Note the change in orientation of the strand indicated by the arrow. (g) 135 min. Dissolution was apparently complete when the particle was viewed microscopically, but the micrograph shows some material remaining (at arrows). The outline of the orignial particle has been preserved by the kaolinite which adhered to its surface. (All photographs are 230X.)

washed once with sterile distilled water, and each was resuspended in sterile distilled water (2.0 mg/ml) for use in tests of enzyme activity. When the second samples were removed from the centrifuge, the supernatant fluids, (designated KS_2, IS_2, BS_2) were decanted, samples were removed for protein estimation and tests of enzyme activity. The particulates (K_2, I_2, B_2) were not washed, but were resuspended in distilled water (2 mg/ml) for enzyme assay. One ml of each sample, resuspended particulate, supernatant fluid or control CMF was added to 0.25 mg of sterile Azocoll; mixed well, and poured with 5 ml of 2% agar at 50 C. Plates were incubated at room temperature (23-25 C).

Table 7 lists the mean and standard error of the mean of the triplicate determinations of protein in each sample fluid. Fig. 34 represents graphically the rate of enzymatic release of Azocoll chromophore by the various particulate fractions and fluids tested. Inspection of Table 7 shows that apparent protein content decreased with the first addition of particulate, but increased with addition of the second sample of particulate. This confirms the statement which we made earlier, that the Folin procedure (Lowry method) is not too reliable when samples have contained samples of particulate material. Control tubes containing no enzyme, but an equivalent amount of particulate; or of enzyme preparations, particulate but no Folin reagents; both have shown that the increase in absorbance is not caused by turbidity of residual mineral. We have concluded that it must be due to reaction between the Folin reagents and some component of the minerals. The effect is less serious with kaolinite, but is of sufficient magnitude with illite and bentonite to render the Folin estimation worthless.

Presence of enzymatic activity on the mineral particulates is, in itself, an indication that protein has been removed from the CMF solution. In this test, we purposely kept the level of particulate below that previously determined as sufficient to remove essentially all protein from solution. This was to "saturate" the mineral with protein, and a proportionately high level of available enzyme, to give optimal conditions for testing the residual enzyme adhering to the particulates after washing. Fig. 34 shows, as expected, that the level of enzyme in KS_2, IS_2, and BS_2 was still high; nearly that of the untreated CMF. Fig. 34 also shows clearly that washed kaolinite retains much more enzyme than washed illite or bentonite. This is probably the reason why kaolinite has been more effective as an "enzyme carrier" than the other minerals, even though bentonite lessened the enzyme activity of CMF to a greater extent, showing that it had removed more enzyme from solution.

Effect of pH on Enzyme Activity

Four centrifuge tubes, each containing 40 mg of kaolinite were sterilized. To each of the first two, 14 ml of CMF from a 4 day culture of S-1 in GM were added. To the third and fourth tubes, 0.01M Tris buffer, pH 7.3, and distilled water, respectively, were added. All tubes were agitated to suspend the kaolinite, then centrifuged. The supernatant fluids were decanted into sterile culture tubes. The supernatant CMF and water, as well as the pellets, from tubes 2 and 4 were frozen and used the next day. The pellets from tubes 1 (CMF) and 3 (Tris buffer) were each washed in 10 ml of buffer. The wash buffers were collected aseptically and tested for enzymatic activity.

The washed kaolinite pellets from tubes 1 (CMF) and 3(buffer) were each resuspended

TABLE 7

Estimation of protein (Lowry method) in cell-free filtrate of Streptomyces sp. S-1 culture medium (CMF); in supernatant CMF (KS_1, IS_1, and BS_1) after addition and removal of the mineral particulates kaolinite (K), illite (I), and bentonite (B); and in supernatant fluids (KS_2, IS_2, and BS_2) after addition and removal of a second sample of the same mineral particulate.

SAMPLE	ESTIMATED PROTEIN CONCENTRATION (µg/ml)	STANDARD ERROR OF THE MEAN
CMF	102	.09
KS_1	91	2.51
KS_2	99	3.17
IS_1	102	2.90
IS_2	103	2.00
BS_1	96	3.33
BS_2	103	1.80

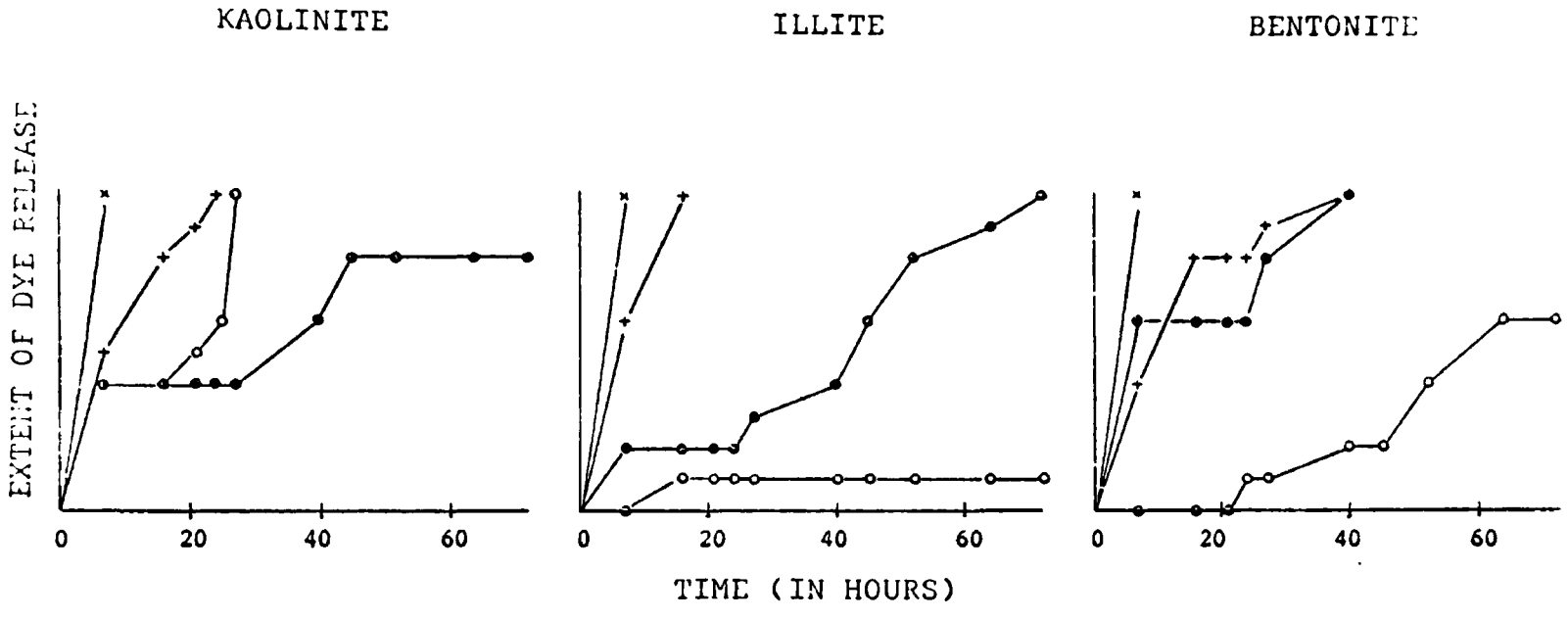

Fig. 34. -- Adsorption of active enzyme to kaolinite, illite and bentonite. Mineral particulates were added to a cell-free filtrate (CMF) of chemically defined medium in which Streptomyces sp. S-1 had been cultured. Enzyme activity was assayed: in CMF (x); on mineral particulates added to and removed from CMF, then washed with distilled water (o); on mineral particulates added to and removed from the supernatant CMF from which the first particulates had been removed (●); in the supernatant CMF after removal of the second particulate sample (+).

in 10 ml of buffer. Eight 1 ml samples of each suspension were distributed to centrifuge tubes and the kaolinite resedimented. The buffer washes from each set of eight tubes were pooled and assayed for enzyme activity. No activity was detectable. Despite the fact that one portion of kaolinite had been in contact with CMF, apparently no enzyme was eluted by the buffer wash. (Several other experiments confirmed this result. Once adsorbed, enzyme remained on kaolinite through several washes with Tris buffer in pH range 7.6 - 8.3).

The kaolinite pellets, now distributed in 16 tubes each containing about 4 mg of mineral, were each resuspended in 5 ml of 0.01M buffer containing Tris-maleate, phosphate, and acetate anions, with H^+, Na^+ and K^+. One kaolinite pellet from CMF and a control pellet from Tris buffer were resuspended in 5 ml of buffer at each of the following pH's: 1.8, 3.0, 4.0, 5.0, 6.0, 7.0, 8.3, and 9.2.

The kaolinite from CMF was difficult to resuspend at the pH's below 7. After the kaolinite had been thoroughly mixed with buffer, the tubes were centrifuged and supernatant buffers decanted for enzyme assays. No enzyme activity was detectable in any supernatant buffer.

The kaolinite pellets from buffers at pH 1.8 - 5.0 were resuspended in Tris buffer and the pH restored to 7.0. The kaolinite was sedimented again. Supernatant buffers were assayed, but evidenced no enzyme activity.

The 16 kaolinite pellets were assayed for enzyme activity. No detectable dye release or dissolution of collagen occurred in plates prepared with CMF-kaolinite exposed to the 3 lowest pH's (1.8, 3.0, and 4.0). There was slight activity in the CMF-kaolinite from buffer at pH 5. In the other preparations, enzyme activity was detectable but was low.

To assay the activity of the original CMF, the supernatant fluids and pellets from tubes 2 and 4 were thawed and the pellets resuspended in Tris buffer. CMF-kaolinite carried much more activity than had the pellet in the mixed buffers tested the previous day. From the procedure used, it was not possible to determine whether loss of activity was due to failure of enzyme to adsorb to kaolinite, elution of inactive enzyme by the mixed buffer at the lower pH's, or inactivation of adsorbed enzyme. The total concentration of the original CMF (about 350 μg/ml) was too low for detection of protein at the dilutions effected by dividing the kaolinite into so many samples and resuspending each sample in buffer.

This experiment did indicate that freezing and thawing kaolinite-adsorbed enzyme did not destroy enzymatic activity.

To circumvent the possibility that enzyme was inactivated by an effect of the Tris-maleate, phosphate, acetate buffer itself, rather than by the pH factor, we adjusted the pH of samples of CMF to 5, 6, 7, 8, 9, 10, filtered the samples to sterilize them, and added sterile kaolinite (6 mg/ml). We removed a 1 ml portion of each sample to determine the pH after addition of kaolinite. Actual pH's were 5.4, 5.6, 7.0, 7.6, 8.4, and 9.2. Results of assays showed that at all pH's the enzyme activity was greater than it had been at corresponding pH's in the mixed buffer. In CMF activity was greatest at pH 9.2. The activity was equivalent at pH's 7.0, 7.6 and 8.4, but somewhat less than at pH 9.2. There

was slight activity exhibited by enzyme adsorbed at pH's 5.4 and 5.6.

This procedure did not differentiate between inactivation of adsorbed enzyme in an acid medium and failure of enzyme to adsorb to kaolinite at low pH's, but the former possibility seems more probable. At low pH, proteins are more cationic and bind more strongly to clay minerals. With an increase in number of potential binding sites, there may have been greater deformation of enzyme configuration, resulting in loss of activity.

Concentration of Enzyme from Dilute Solution

To a CMF solution containing about 350 μg of total protein per ml, we added 2.5 mg/ml of kaolinite. Previous tests had indicated that this ratio of kaolinite to protein should reduce the protein content of the solution below detectable levels. The kaolinite was removed by centrifugation. Samples of the supernatant CMF and of the kaolinite pellet were assayed for proteolytic activity. In the remainder of the supernatant CMF, we suspended an additional 1 mg/ml of kaolinite and assayed the suspension for enzyme activity. Fig. 35 is a flow diagram of the procedure, and indicates the resultes. There was much enzyme activity in the first kaolinite sample, and virtually none in the supernatant CMF. But addition of kaolinite to the supernatant CMF made residual enzyme in that solution detectable. When supernatant CMF was mixed with HPA and poured in agar, what enzyme there was, was distributed in 5 ml of agar. Presumably kaolinite, added to the solution, concentrated the enzyme and as the kaolinite adhered to HPA particles, effected localization of the enzyme on substrate surfaces. This would prevent dispersal of the enzyme throughout the agar.

DISCUSSION

Interactions among microbial cells and their extracellular enzymes, particulate substrates and clay minerals have been studied from several aspects, but no comprehensive or coherent view of the subject can be synthesized from the literature. Clay minerals have been found to influence microbial metabolism in in vitro studies, but the mode of influence has been attributed to different mechanisms by different authors and probably does differ with changes in the microbes, substrates and minerals involved.

The role of clay minerals in the metabolism of soil organisms has been extensively investigated, but in considerations of aquatic microbial activity, the presence of mineral particulates in the milieu has, until recently, been largely ignored.

In attempting to form some picture, based on the available literature, of the array of interdependent interactions which must occur during the degradation of complex insoluble organic materials by lake organisms, the following conditions could be postulated as a null hypothesis:

1. The mycelia of lake dwelling actinomycetes such as Streptomyces and Micromonospora (reported to utilize collagen, chitin and cellulose, as well as many other materials such as keratin and lignin) are normally covered with an adherent layer of fine clay particles, and may grow attached to larger particulate surfaces (the way they adhere to laboratory

CONCENTRATION OF ENZYME BY KAOLINITE

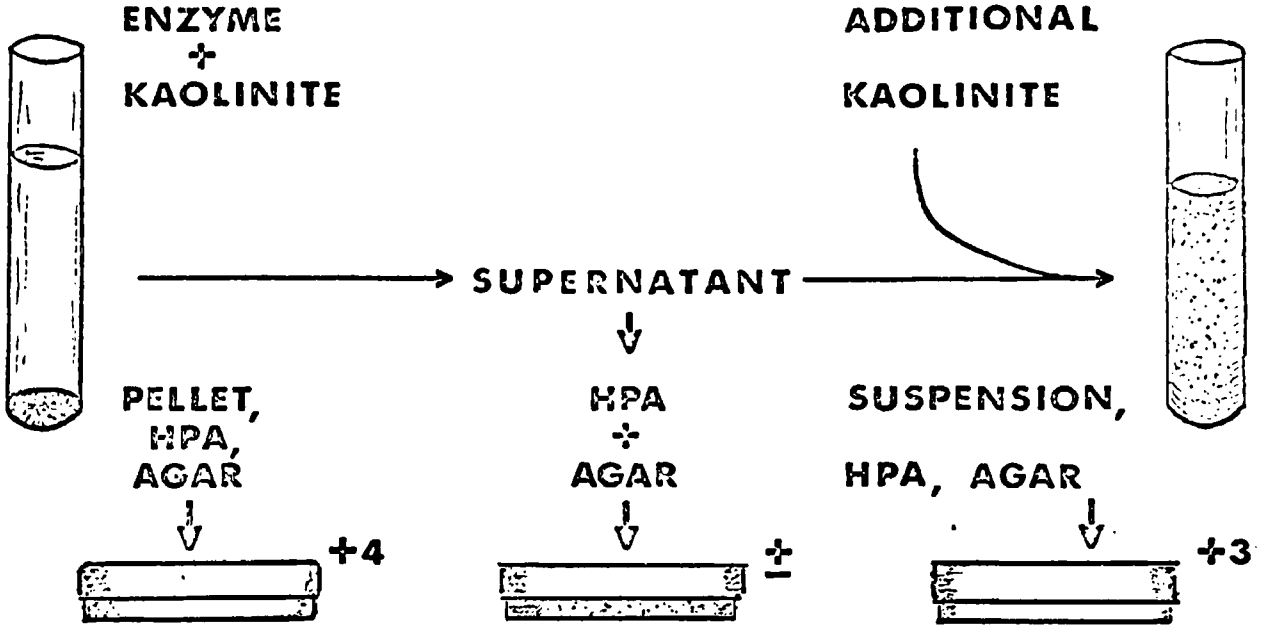

Fig. 35. -- Concentration of protease from Streptomyces sp. S-1 culture medium filtrate by kaolinite. Kaolinite (2.5 mg/ml) was added to culture medium filtrate (CMF) and removed by centrifugation. The kaolinite and supernatant fluid were assayed for enzyme activity, which was high on the kaolinite but very low in the supernatant fluid. Addition of 1 mg/ml of kaolinite to the supernatant CMF concentrated the enzyme and made its detection possible.

culture flask walls).

2. Fortuitous collisions between organisms and particulate substrates may result in the formation of temporarily stable aggregates, the stability mediated partially by adherent mineral species, and lasting as long as the substrate lasts.

3. Extracellular enzymes produced by organisms do not diffuse into the surrounding water, but are adsorbed to substrate and mineral surfaces.

4. The combination of these interactions has, for aquatic microorganisms, converted a potentially haphazard method of degrading substrates and recovering the components, into an efficient means of recycling organic compounds and acquiring nutrients.

To determine whether there is, in fact, any basis for making these assumptions, we have isolated and studied some aspects of each constituent interaction involved in the network comprising the null hypothesis.

For preliminary studies, to determine appropriate techniques, we worked with laboratory strains of S. fradiae and M. chalcea. They did utilize collagen and chitin readily, and cellulose slowly. The mineral particulates, kaolinite and bentonite, adhered to the mycelia of both organisms with enough tenacity to withstand separation on a water: 65% (w/v) sucrose density gradient. Protein released into chemically defined culture media by M. chalcea was adsorbed by kaolinite and bentonite. Although the interference by mineral particulates prevented precise determination by the Folin method, of the amount of protein removed from solution by the particulates, patent enzymatic activity located on the particulates, even after washings, demonstrated adequately that some protein was adsorbed to the mineral surfaces. Results obtained with HPA and Azocoll proved that dye release from substrate conjugated to a chromophore was a sensitive indicator of enzyme activity. Spectrophotometric quantitation of released dye was impractical in systems containing mineral particulates because the chromophore adhered to the mineral, but this very situation served to illustrate that small molecules released from the substrate by enzyme activity were adsorbed to the local mineral particulates. The proteolytic activity evidenced by kaolinite and bentonite removed from culture medium containing too little enzyme to effect any decolorization of Azocoll without the mediation of mineral particulates, illustrated the way in which minerals may effect contact between enzymes and substrates occurring extremely dilute environments.

To determine whether the sorts of results obtained with S. fradiae and M. chalcea could be achieved with organisms indigenous to a lake, we isolated three strains of Streptomyces and four of Micromonospora from Lake Erie and tested them for substrate utilization. Of the seven strains, two streptomycetes and one micromonospora cultured well only on media containing casein; starch and casein; tryptone and malt extract, or yeast and malt extract. (This Micromonospora grew poorly and was not tested on limited media.) One streptomycete and two micromonosporas far outstripped their laboratory counterparts in the rapidity with which they proliferated on the entire array of substrates tested. This seems to leave no doubt that actinomycetes capable of recycling plant and animal debris do exist in Lake Erie.

One very significant result of these substrate utilization tests was the unequivocal demonstration that the streptomycete designated S-1 was capable of degrading native collagen - a product resistant to tryptic hydrolysis. Since most methods used to sterilize collagen denature it to a trypsin sensitive form, many claims of collagenase activity are dubious; a general protease may have effected the reported results.

The extraordinary growth of several actinomycete strains in a salts medium containing chitin remains unexplained. If a growth promoting factor is present as a contaminant in the crude chitin preparation, it is not soluble, and is not a nitrogen or carbon source per se.

S-1 proved to utilize cellobiose and cotton fiber cellulose. On agar plates, S-1 not only produced cellulase (s) capable of completely hydrolyzing the fibers within six weeks, but also penetrated the agar to a depth of 4 to 5 mm and formed colonies completely filling the spaces vacated by dissolution of the cellulose. It may prove disconcerting to taxonomers to learn that while we were examining the extent of cellulose degradation by S-1, we found chains of conidia formed in mycelia embedded in agar 4 to 5 mm below the surface.

Five interactions involving clay minerals occurred. Adherence of kaolinite, illite and bentonite to S-1 mycelia suspended in culture medium at a series of pH's from 6 to 10 was demonstrated photographically. The extent of adherence varied with pH and was not uniform among the minerals used, but at all pH's tested, aggregation of mineral and cells did occur. The most important fact was that cell-mineral interactions were greatest at the pH's encountered under normal lake conditions.

Tests of sedimentation velocity demonstrated the same phenomenon: interaction among cells and kaolinite was greatest at pH 8.0 - 9.0. The pH of the water and mud sample used to isolate organisms from Lake Erie was 8.4. Data from the sedimentation tests show that the amount of settling of cells was negligible during a 30 min period, and was little affected by change of pH. Sedimentation of kaolinite was rapid at all pH's but the rate varied significantly among those pH's tested. This could be expected since charge sites at kaolinite surfaces influence the edge-to-face attraction among crystals. The sample of kaolinite which we used was self-flocculating at pH 8.0 - 8.5. The tremendous significance of these data lies in the difference between sedimentation rates of cells and of cell-kaolinite aggregates. All the data obtained demonstrate overwhelmingly that cells and minerals do aggregate at the pH range normal to Lake Erie mud. These aggregates are stable, withstanding separation by density gradient centrifugation. Kaolinite also formed stable complexes with blood fibrin and collagen, greatly increasing the densities of the proteins. All of these data suggest that in water containing suspended clay minerals, bacterial cells and particulate protein substrates can be coated with mineral particles and would settle at rates largely influenced by the density of the minerals. Cell-substrate interactions, then would occur mainly in the bottom waters and sediments.

All three minerals tested removed enzyme from solution. In all cases, some enzyme was in active form. Kaolinite proved able to concentrate enzyme from solutions too dilute to exhibit any activity without the mediation of the mineral. Adsorption of enzyme to kaolinite and subsequent adherence of the kaolinite to collagen resulted in enhancement of apparent enzyme activity, presumably by bringing the enzyme and substrate into intimate contact.

One implication of these results is somewhat disquieting. The effects of mineral particulates significantly altered cell densities, enzyme concentrations and enzyme reaction rates. Typical laboratory studies of microbial enzyme activities may hardly describe the actual events which occur in the presence of clay minerals. To assume, for example, that microbial activity in a lake may be measured by studying the activity of organisms sampled from the water column, may be grossly to underestimate the true activity.

Adsorption of organic matter on clay minerals is probably a mechanism with evolutionary advantages. Bernal (8) suggested that it was on the surface of kaolinite that the first dilute organic materials were concentrated sufficiently to allow for the formation of self-replicating units. Aggregation of substrates, cells and enzymes on mineral surfaces optimizes the organism's ability to erode nutirents from the surfaces of resistant materials, and concomitantly returns the components of organic detritus to the nutrient pool.

Since clay minerals are implicated in the natural processes which recycle organic materials (30), investigation of their use in waste water treatment seems worthwhile. The rapidity with which the protease of S-1 disposed of visible sized particles of Azocoll suggests that mineral-adsorbed enzyme could be used to degrade selected waste materials into usable amino acids or carbohydrates. The use of enzymes linked to cellulose derivatives has proven feasible (6, 27, 28, 36, 37, 55). The enzymes are insolubilized and reusable.

The potency of actinomycete enzymes has received little attention. Nomoto and Narahashi (59) reported that a protease of S. griseus was as active as the Bacillus subtilis enzymes usually used commercially. Culture media from antibiotic and vitamin producing actinomycetes might prove to be a source of useful enzymes.

In the interactions among cells, enzymes, substrates, and clay minerals points of agreement or disagreement with previous workers are hard to determine. Because the outcome of any particular interaction seems to vary with slight changes in many factors, comparisons between our results and those of any other worker are almost impossible to make. Since we were not studying the effects of minerals on microbial metabolism as such, our data do not fall into the same categories (metabolic rates, O_2 uptake, length of lag period, total cell mass) as those used by previous authors. Rather, the data should help to explain how some of the observations made by microbiologists can be interpreted in the light of concepts developed in the soil sciences.

SUMMARY

We investigated the interactions among actinomycete cells, their extracellular enzymes, substrates and clay minerals to determine what influence mineral particulates might have on microbial degradation of complex organic substrates.

Laboratory strains of Actinomyces and one Lake Erie isolate of Streptomyces could be cultured in a chemically defined salts medium containing any of the following carbon and nitrogen sources: glucose L- asparagine + ammonium acetate; cellulose + ammonium acetate; collagen, or chitin. Five strains of actinomyces isolated from Lake Erie rapidly

degraded autoclaved collagen, chitin and cellulose. Mycelia enveloped the substrate particles or formed colonies on substrate surfaces.

Mineral particulates adhered to actinomycete mycelia with enough force to resist separation by density gradient centrifugation at 13,200 x g for 1 hr. Adherence of minerals occurred at pH's 6.0 - 10.0; the extent varying with mineral species and pH. Cell age, and the concentration of the suspending buffer, significantly influenced the sedimentation rate of cell-kaolinite aggregates.

Density gradient centrifugation demonstrated adherence of kaolinite to Hide Powder Azure (HPA).

Kaolinite, illite and bentonite adsorbed protein from cell-free filtrates (CMF) of chemically defined medium in which actinomycetes had been cultured. Assay of CMF before addition of mineral; and of the supernatant CMF and sedimented mineral after removal by centrifugation, demonstrated adsorption of active enzyme by minerals. Distilled water washing reduced residual enzymatic activity on minerals in the order: illite > bentonite > kaolinite. Enzyme activity on minerals was low at pH's 5 - 7, anc could not be detected at pH's below 5.

Proteolytic enzymes in solution were assayed by spectrophotometric measurement of azo chromophore released from Azocoll or (HPA). Enzyme adsorbed to mineral particulates was assayed by allowing the mineral to adhere to HPA, then pouring the aggregates with agar. Dye release and dissolution of HPA structure could be estimated visually and recorded photographically.

Presence of proteolytic enzyme in solutions too dilute to evidence activity by HPA-agar plate assay, could be detected by adding kaolinite to the solutions, removing the mineral and mixing it with HPA. The kaolinite apparently adsorbed enzyme, and by adhering to HPA, brought the enzyme in contact with the substrate.

BIBLIOGRAPHY

1. Adams, M. and C. S. Hudson. 1943. New methods for the purification of invertase and some properties of the resulting products. J. Am. Chem. Soc. 65: 1359-1368.
2. Ambroz, Z. 1966. Action of bentonite on the microbial decomposition of proteins in soil. Sbornik Vysoke Skoly Zemedelske. Brne, Rada, A 1966: 401-406.
3. Bader, R. G., D. W. Hood, and J. B. Smith. 1960. Recovery of dissolved organic matter in sea water and organic sorption by particulate material. Geochim. Cosmochim. Acta 19: 236-243.
4. Bader, R. G. and L. M. Jeffrey. 1958. The exchange capacity, organic adsorption and differential flocculation of clay minerals as related to radioactive waste disposal. Part V, Texas A and M Research Foundation, Project 142 Annual Report, 1-25.
5. Barbaro, R. D. and J. V. Hunter. 1965. Effect of clay minerals on surfactant biodegradability. Purdue Univ. Eng. Bull. Ext. Serv. No. 118: 189-196.
6. Bar-Eli, A. and E. Katchalski. 1963. Preparation and properties of water insoluble derivatives of trypsin. J. Biol. Chem. 238: 1690-1698.
7. Berger, L. R. and D. M. Reynolds. 1958. The chitinase system of a strain of Streptomyces griseus. Biochim. Biophys. Acta. 29: 522-534.
8. Bernal, J. D. 1967. The origin of life. The World Publishing Co. Cleveland, Ohio. 345p.
9. Bigger, J. W. and J. H. Nelson. 1941. The growth of coliform bacteria in distilled water. J. Path. Bact. 53: 189-206.
10. Black, A. P., F. Birkner, and J. J. Morgan. 1966. The effect of polymer adsorption on the electrokinetic stability of dilute clay suspensions. J. Colloid Interface Sci. 21: 626-648.
11. Buckman, H. O. and N. C. Brady. 1960. The nature and properties of soils. 6th ed. The Macmillan Co. New York. 567p.
12. Busch, P. L. and W. Stumm. 1968. Chemical interactions on the aggregation of bacteria: bio-flocculation in waste treatment. Environ. Sci. Technol. 2: 49-53.
13. Button, D. K. 1969. Effect of clay on the availability of dilute organic nutrients to steady state heterotrophic populations. Limnology Oceanogr. 14: 95-100.
14. Cashen, G. H. 1966. Electric charges of clays. J. Soil Sci. 17: 303-316.
15. Conn, H. J. and Conn, J. E. 1940. The stimulating effects of colloids upon the growth of certain bacteria. J. Bacteriol. 39: 99-100.
16. Cralley, K. E. 1968. The effect of particulates on the growth of selected actinomycetes. M. S. Thesis. Ohio State University. 90 p.
17. Dixon, M. and K. Kodama. 1926. Purification of xanthine oxidase. Biochem. J. 20: 1104-1110.
18. Enger, M. D. and B. P. Sleeper. 1965. Multiple cellulase system from Streptomyces antibioticus. J. Bacteriol. 89: 23-27.
19. Ensminger, L. E. and J. E. Gieseking. 1939. The adsorption of proteins by montmorillonitic clays. Soil Sci. 48: 467-473.
20. Ensminger, L. E. and J. E. Gieseking. 1942. Resistance of clay-adsorbed proteins to proteolytic hydrolysis. Soil Sci. 53: 205-209.
21. Erikson, D. 1941. Studies on some lake-mud strains of Micromonospora. J. Bacteriol. 41: 277-303.
22. Esterman, E. F. and A. D. McLaren. 1959. Stimulation of bacterial proteolysis by adsorbants. J. Soil Sci. 10: 64-78.

23. Esterman, E. F., G. H. Peterson, and A. D. McLaren. 1959. Digestion of clay-protein, lignin-protein and silica-protein complexes by enzymes and bacteria. Soil Sci. Soc. Am. Proc. 23: 31-36.
24. Evans, L. T. and E. W. Russel. 1959. The adsorption of humic and fulvic acids by clays. J. Soil Sci. 10: 119-132.
25. Fraser, M. J. Behavior of Proteins at interfaces. Jour. Pharmacy and Pharmacology 9: 497-521.
26. Gallop, P. M., S. Seifter, and E. Meilman. 1959. Studies on Collagen. I. The partial purification, assay, and mode of activation of bacterial collagenase. J. Biol. Chem. 227: 891-906.
27. Goldstein, L. and E. Katchalski. 1968. Use of water-insoluble enzyme derivatives in biochemical analyses and separation. Z. Anal. Chem. 243: 375-396.
28. Goldstein, L., Y. Levin and E. Katchalski. 1964. A water insoluble polyanionic derivative of trypsin. II. Effect of the polyelectrolyte carrier on the kinetic behavior of the bound trypsin. Biochem. 3: 1913-1919.
29. Gomori, G. 1955. Preparation of buffer for use in enzyme studies. p. 138-146 In Colowick, S. P. and N. O. Kaplan (ed), Methods in Enzymology. Vol. I. Academic Press, New York. 835 p.
30. Hagen, P., E. T. Reese, and O. A. Stamm. 1966. Zur Reaktion von Reaktivfarbstoffen mit Cellulose. IV. Enzymatische Hydrolyse reactiv gefärbter Cellulose. Helv. Chim. Acta 49: 2278-2286.
31. Hagihara, B. 1960. Bacterial and mold proteases. p. 193-213. In Boyer, P. D., H. Lardy and K. Myrback, (ed) The Enzymes. Vol. 4 Academic Press, New York.
32. Heller, W. and T. L. Pugh. 1960. Steric stabilization of colloidal solutions by adsorption of flexible macromolecules. J. Polymer Sci. 47: 203-217.
33. Hendricks, S. B. 1941. Base exchange of the clay mineral montmorillonite for organic cations and its dependence upon adsorption due to van der Waals forces. J. Phys. Chem. 45: 65-81.
34. Hendricks, C. W. 1971. Enteric bacterial metabolism of stream sediment eluates. Can. J. Microbiol. 17: 551-556.
35. Heukelekian, H. and A. Heller. 1940. Relation between food concentration and surface for bacterial growth. J. Bacteriol. 40: 547-558.
36. Hornby, W. E., M. D. Lilly and E. M. Crook. 1966. Preparation and properties of ficin chemically attached to carboxymethyl-cellulose. Biochem. J. 98: 420-425.
37. Hornby, W. E., M. D. Lilly, and E. M. Crook. 1968. Some changes in reactivity of enzymes resulting from their chemical attachment to water-insoluble derivatives of cellulose. Biochem. J. 107: 669-674.
38. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bact. Rev. 14: 1-49.
39. Jagnow, G. 1957. Beiträge zur Ökologie der Streptomyceten. Arch. Mikrobiol. 26: 175-191.
40. Jensen, H. L. 1930. Actonomycetes in Danish soils. Soil Sci. 30: 59-77.
41. Kitchener, J. A. 1969. Colloidal minerals: chemical aspects of their dispersion, flocculation and filtration. Filtration and Separation 6: 553-558.
42. Lee, G. F. and A. W. Hoadley. 1967. Biological activity in relation to the chemical equilibrium composition of natural waters. p. 319-338. In W. Stumm (Chairman). Equilibrium concepts in natural water systems: a symposium. Adv. Chem. 67. American Chemical Society, Pittsburgh, Pa. 344p.

43. Lowry, O.H., N.J.Rosebrough, A.L.Fare, and R.J.Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
44. Lynch, D.L., L.M.Wright, and L.J.Cotnoir, Jr. 1956. Adsorption of carbohydrates and related compounds on clay minerals. Soil Sci. Soc. Am. Proc. 20: 6-9.
45. Mandel, I. 1961. Collagenases and elastases. Advances Enzymol. 23: 163-264.
46. Mandels, M. and J.Weber. 1969. The production of cellulases. p. 391-414. In. R.F. Gould (ed) Advances in chemistry series, No. 95. Am. Chem. Soc., Washington, D.C.
47. Manual of Microbiological Methods. 1957. McGraw-Hill Book Company, Inc. New York.
48. Mattson, S. 1932. The laws of soil colloid behavior: VII. Proteins and proteinated complexes. Soil Sci. 33: 41-72.
49. McCabe, P.A. and J.I.Frea. 1971. Interaction of a clay mineral, microbial cells and enzymes in the degradation of solid organic materials. Abstr. 14th Conf. Great Lakes Res. 1971: 34-35.
50. McLaren, A.D. 1954a. The adsorption and reactions of enzymes and proteins on kaolinite. I. J. Phys. Chem. 58: 129-137.
51. McLaren, A.D. 1954b. Adsorption and reactions of enzymes and proteins on kaolinite. II. The action of chymotrypsin on lysozyme. Soil Sci. Soc. Am. Proc. 18: 170-175.
52. McLaren, A.D. 1957. Concerning the pH dependence of enzyme reactions on cells, particulates, and in solution. Science 125: 697.
53. McLaren, A.D., G.H.Peterson, and I.Barshad. 1958. Adsorption and reactions of enzymes and proteins on clay minerals: IV. Kaolinite and montmorillonite. Soil Sci. Soc. Am. Proc. 22: 239-244.
54. Meadows, P.S. and G.J.Anderson. 1966. Microorganisms attached to marine and freshwater sand grains. Nature 212: 1059-1060.
55. Mitz, M.A. and L.J.Summaria. 1961. Synthesis of biologically active cellulose derivatives of enzymes. Nature. 189: 576-577.
56. Mukherjee, S.K. and S.K.Chakravarti. 1968. Sedimentation volume and viscosity of pure clay minerals and their mixtures as influenced by addition of quarternary ammonium compounds. J.Ind. Chem. Soc. 44: 167-174.
57. Narksi, N., M.Rebhun, and H.Sperber. 1968. Flocculation of clay suspensions in the presence of humic and fulvic acids. Israel J. Chem. 6: 295-305.
58. Nelson, W.L., E.I.Ciacco, and G.P.Hess. 1961. A rapid method for the quantiative assay of proteolytic enzymes. Anal. Biochem. 2: 39-44.
59. Nomoto, M. and Narahashi, Y. 1959. A proteolytic enzyme of Streptomyces griseus. J. Biochem. 46: 653-667.
60. Nováková, J. 1968. Effect of bentonite and kaolinite on the growth curve of E. coli. Folia Microbiol. 13: 543.
61. Oakley, C.L., G.H.Warrack, and W.E. Van Heyningen. 1946. The collagenase (K toxin) of Cl. Welchii type A. J. Path. Bact. 58: 229-235.
62. Pfister, R.M., P.R.Dugan, and J.I. Frea. 1968. Particulate fractions in water and the relationship to aquatic microflora. Proc. 11th Conf. Great Lakes Res. 1968: 111-116. International Assoc. Great Lakes Res.
63. Pinck, L.A. and F.E.Allison. 1951. Resistance of protein-montmorillonite complex to decomposition by soil microorganisms. Science 114: 130-131.
64. Pridham, T.G., P.Anderson, G. Foyle, L.A.Lindenfelser, C.W.Hesseltine, and R.G.Benedict. 1956-57. A selection of media for maintenance and taxonomic study of streptomycetes. Antibiotics Ann. 1956-1957: 947-953.

65. Pridham, T.G. and D.Gottlieb. 1948. The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107-114.
66. Reese, E.T., R.G.H.Siu, and H.S.Levinson. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59: 485-497.
67. Reese, E.T., E.Smakula, and A.S.Perlin. 1959. Enzymic production of cellotriose from cellulose. Arch. Biochem. Biophys. 85: 171-175.
68. Reynolds, D.M. 1954. Exocellular chitinase from a Streptomyces sp. J. Gen. Microbiol. 11: 150-159.
69. Rinderknecht, H., M.C.Geokas, P.Silverman, and B.J.Haverback. 1968. A new ultrasensitive method for the determination of proteolytic activity. Clin. Chim. Acta. 21: 197-203.
70. Ruehrwein, R.A. and D.D. Ward. 1952. Mechanism of clay aggregation by polyelectrolytes. Soil Sci. 73: 485-492.
71. Skujins, J.J., H.J.Potgieter, and M.Alexander. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and $\beta\beta$ -(1→3) glucanase. Arch. Biochem. Biophys. 111: 358-364.
72. Smith, N.R. and V.T.Kawson. 1944. The bacteriostatic action of rose bengal in media used for plate counts of soil fungi. Soil Sci. 58: 467-471.
73. Stotzky, G. 1966a. Influence of clay minerals on microorganisms. II. Effect of various clay species, homoionic clays and other particles on bacteria. Can. J. Microbiol. 12: 831-848.
74. Stotzky, G. 1966b. Influence of clay minerals on microorganisms. III. Effect of particle size, cation exchange capacity, and surface area on bacteria. Can. J. Microbiol. 12: 1235-1246.
75. Stotzky, G. and L.T.Rem. 1966. Influence of clay minerals on microorganisms. I. Montmorillonite and kaolinite on bacteria. Can. J. Microbiol. 12: 547-563.
76. Stotzky, G. and L.T.Rem. 1967. Influence of clay minerals on microorganisms. IV. Montmorillonite and kaolin on fungi. Can.J. Microbiol. 13: 1535-1550.
77. Talibudeen, O. 1950. Interlamellar adsorption of protein monolayers on pure montmorillonoid clays. Nature 166: 236.
78. Talibudeen, O. 1955. Complex formation between montmorillonoid clays and amino-acids and proteins. Trans. Faraday Soc. 51: 582-590.
79. Ulinska, A. and L.Huppenthal. 1966. The stabilization and aggregation effect of polyelectrolytes on suspensions. II. The influence of polymetharylic acid on water suspensions of kaolinite and active carbon. Stud. Soc. Sci. Torun., Sec. B 4: 37-50.
80. Waksman, S.A. 1967. The Actinomycetes: a summary of current knowledge. Ronald Press, New York. 280 p.
81. Wayman, C.H. 1967. Adsorption on clay mineral surfaces. p. 127-167. In Faust, S.D., and J.V.Hunter (ed) Principles and Applications of Water Chemistry. Proc. 4th Rudolphs Res. Conf. Rutgers, The State University, New Brunswick, N.J. John Wiley and Sons, Inc., New York. 643 p.
82. Weber, J.B. and H.D.Coble. 1968. Microbial decomposition of diquat adsorbed on montmorillonite and kaolinite clays. J. Agr. Food Chem. 16: 475-478.
83. ZoBell, C.E. 1943. Effect of solid surfaces upon bacterial activity. J. Bacteriol. 46: 39-56.

Part 6

Suspended particles from Lake Erie: Amino acid composition and the effect of detergents on their interactions with bacteria.

Part 6

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The microparticulate material in lake water may participate in microbial interactions in several ways. The particulates may serve as a surface for enzymatic reactions, thereby increasing their efficiency in dilute solutions (McLaren, 1963). They may act as interfaces for the entrapment of nutrients (Zobell, 1943). The particulate material may itself be utilized as a nutrient source (Jorgensen, 1967). Finally, as these particulates and microorganisms form larger and larger aggregates, they settle out forming a portion of the bottom sediments.

Surface-active agents such as detergents have a high affinity for interfaces (Renn, 1963). It is possible that the reduction of the interfacial tension at the surfaces of these particulates would cause the release of some bound nutrients or harmful products. The accelerated release of nutrients or harmful products, such as pesticides, into the microenvironment, could have a serious effect on the ecology.

This research was undertaken to study the role of the particulate material in Lake Erie with emphasis placed on the ability of these particulates to serve as sources of carbon or nitrogen for growth. Any suspended matter, from 0.02μm (colloidal size) to 10μm, was considered as particulate. The chemical composition of the particulates was analyzed in an attempt to correlate the information obtained with the observed patterns of utilization. Experiments were performed to determine the effects of surfactants on microbial-particulate interactions.

REVIEW OF THE LITERATURE

Composition of the Particulate Material

Physical Parameters

Since there is a fine difference between dissolved and particulate matter, almost every author has his own arbitrary scheme of classification. Some classify unsettleable solids as dissolved matter (Duursma, 1960) while others consider all colloidal matter (1 μm - 0.001 μm) as particulates (Fox et al., 1953). Other investigators place a 0.5 μm lower limit on particulate material (Krogh, 1934). Similarly, the upper limit of particle size is indeterminate with estimates ranging as high as 1-3 mm in sea water. The general methods of collection have been filtration with a 0.5 μm filter, 24 hour settling vats or centrifugation.

Estimates of size for seawater particulates are higher than for those from fresh water. Since many of the particulates are negatively charged, the high ionic content of the sea may induce greater aggregation, and consequently larger particulates. Manheim et al. (1970), in their analysis of particulate sizes in the Atlantic Ocean, have found modal values ranging from 4 to 250 μm.

Particulate material from Lake Erie collected by centrifugation and sized with a Zeiss Particle Size Analyzer (Pfister et al., 1970), has demonstrated a range of sizes from 0.028 μm to 7.9 μm, with the greatest number of particulates in the range of 0.05 to 0.2 μm. Thus, in fresh water the mean size of the particulates is within colloidal limits.

Inorganic Components

Clay minerals form the main portion of the inorganic particulate material. Recorded values for the percentage clay range from 64 to 86% of the particulate material in the oceans (Lisitzen, 1959; Postma, 1954). Both their prevalence and their ability to adsorb or retain ions and molecules accounts for their importance. In the presence of cations some of the negative charges on these clays are neutralized, and larger and larger aggregates form. It is these larger aggregates that interact more readily with the larger bacterial cells (Brock, 1966). Clay minerals also adsorb some anions, such as phosphate, very readily, while others such as nitrates show essentially no adsorptive capacity (Lee, 1970).

Organic Analyses

Carbon - Most of the analyses of organic carbon content of particulates have been performed on seawater. Many authors consider phytoplankton to be the main source of living organic carbon (Parsons, 1963). Attempts have been made to represent average carbon values for phytoplankton based on the argument that the major differences in carbon analyses were due to environmental changes. Parsons (1963) compared values for different classes of organics obtained under different environmental conditions. He concluded that while environmental conditions affect changes within the class of organisms, each different class has its own characteristic organic composition. His observations then compound the problem of analysis of these particulates.

Manheim et al. (1970) determined the percent combustible organic matter in surface and subsurface waters along the Atlantic coast. The values they obtained ranged from 24-90% combustible organics in the surface waters and from 50-83% in the subsurface waters. All of the subsurface readings were taken at the one position where the 90% surface sample was found. "Soft gelatinous or flocculent particles, sometimes as large as 1 cm in diameter" were observed in the water column where the extremely high values were obtained. In microscopic observations of the surface waters the authors noted that recognizable organisms or remains of organisms made up only a small portion of the particulate matter despite the high percentage of organic carbon. Irregular organic aggregates and inorganic pollutants were the main particulates noted.

When microbial biomass along the Atlantic Coastline was estimated on the basis of chlorophyll and adenosine triphosphate (ATP) measurements, the surface value obtained was 35 μg C/liter. The value at a depth of 200 m was 5 μg C/liter (Holm-Hansen, 1969). Handa and Tominaga (1969) analyzed particulate material from the coastal waters off Japan for organic carbon content and found that it ranged from 67 μg C/liter at the surface to 30μg C/liter at 200 m depth. Although there was a decrease in values in each set of determinations a much more rapid decrease with increasing depth was noted when measurements were made in terms of living cells.

Handa and Tominaga (1969) also determined the carbohydrate content of the particulates. Acidic hydrolysates revealed the presence of the D-isomers of galactose, glucose, mannose, xylose and glucuronic acid. Determinations of the composition of the particulates at different depths revealed that D-glucose and its polymers were preferentially removed with increasing depth. The particulate carbohydrate material found below 200 m was refractory to attack. Thus, the authors concluded that a homologous distribution of carbon exists below a certain depth in the ocean. Furthermore their analyses suggest that these refractory carbohydrate materials are remnants of structural 1,4-poly saccharide. These and other results lead the authors to the conclusion that deep water particulate material originates from the plankton in the surface waters.

Gordon (1969) has studied the non-living organic particulates in the Atlantic. Microscopic observations and histochemical stains were used to identify the particulates larger than 5 μm. Four types were noted: i) Aggregates containing phytoplankton, bacteria, clays and some indeterminate forms which contained mainly carbohydrates; ii) Flakes, thin scale-like particles with a distinct outline, mainly protein; iii) Fragments, particles formed from the decay of tissue which appeared to be mainly cellulose; iv) unclassifiable particles containing those too large or too small.

One of the few analyses of fresh water particulates was that of Krogh and Lange (1934) on Lake Fureso in Denmark. The particles, removed by ultrafiltration, contained 0.67mg/liter organic matter. Their study showed that only 19% of the particulate colloidal material was organic in nature and was very stable to biological attack.

Nitrogen - Amounts of particulate nitrogen found at different depths in the ocean vary less than carbon. Holm-Hansen (1969) found 4 μg N/liter at the surface and 1.5 μg N/liter at a depth of 200 m in the coastal waters of California, by filtration with a 25 mm glass fiber filter. Handa and Tominaga (1969) collected 5 μg N/liter in the surface waters and 3 μg N/liter at 200 m depth from water off Japan using a 984-H ultrafilter.

Handa and Tominaga found that over 85% of the particulate organic material was proteinaceous in nature. Through comparisons of the relative rates of decay the authors determined that the particulate carbohydrates decayed more rapidly than the proteinaceous amino acids which were utilized at greater depths.

Many researchers have concentrated their analyses on proteinaceous amino acid nitrogen, believing this to be the main organic nitrogenous constituent of the particulate material.

One of the earliest freshwater studies was that of Peterson et al. (1925) on four Wisconsin lakes and consisted of a rapid centrifugation of lake water which removed 75% of the bacteria followed by pan evaporation. These two fractions were classified as planktonic and soluble. There were 92 mg planktonic nitrogen/m^3 and over 400 mg soluble nitrogen/m^3. The free amino acid nitrogen was 54 mg/m^3. Fifty to seventy-five percent of the total soluble nitrogen was found to be organic and over half of this was in amino groups. The free amino acids constituted one-third, the other two thirds being composed of polypeptides. From an analysis of their data on lakes of varying productivity the authors

concluded that the soluble nitrogen is largely dependent on the number of organisms in suspension.

Vallentyne (1954) used paper chromatography to determine the nature of nitrogenous compounds from both fresh and marine waters. In the suspended matter he found cystine, histidine, lysine, tryptophan and tyrosine as free amino acids. In the hydrolysates of the particulate matter, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, leucine, phenylalanine, proline, tryptophan, tyrosine and valine were identified. No free amino acids were detected in the analyses of dissolved organic matter from fresh and marine waters. Vallentyne (1956) concluded that insufficient material had been added to the chromatogram.

Parsons and Strickland (1962) analyzed a single Pacific Ocean sample for the presence of amino acids. They detected glutamic acid, aspartic acid, lysine, arginine, serine, proline, alanine and glycine; and determined that alanine and glycine were the predominant amino acids present in the particulates.

The most extensive analysis of the particulate and free amino acids from sea water was performed by Siegel (1967), who separated particulates by filtration. The particulate amino acids were acid hydrolyzed and analyzed on an amino acid analyzer. The free amino acids were removed from the water column by passage through a Cu-Chelex column. One hundred percent recovery of known amounts of standard amino acids was achieved, with the exception of cysteine. All of the common amino acids except tryptophan were found in the particulate fraction, with arginine, lysine and glutamic acid occurring in the greatest concentration. Sixteen amino acids were found free, not bound as proteins or to particles, as compared with twenty in the particulates. The predominant free amino acids were serine and glycine.

The findings of several investigators indicate that most of the biochemical reactions of particulate matter occur in the upper 200 m of the ocean. Both Holm-Hansen (1969) and Handa and Tominaga (1969) found a fairly stable amount of particulate organic carbon and nitrogen below 200 m. Williams and Gorden (1970) have taken an interesting approach to the question of the stability of these fractions. They reasoned that if the organic particulates were biochemically stable there would be no variance in the $^{13}C/^{12}C$ ratios at different depths below 200 m. Thus, they analyzed the carbon isotope ratios or dissolved organic material and the plankton as well as the particulate organic matter larger than 1 μm. The dissolved and the particulate organics, separated by filtration through a 1 μm glass fiber filter, were found to be very stable when compared to the phytoplankton. The authors suggest that the particulate organic matter is stable below 200 m depth and further that the particulate organic matter is similar in composition to the dissolved organic matter.

Function of the Particulate Material

Although it has been known for a long time that bacterial populations increase during storage it was not until the work of Zobell (1943) that there was any understanding of this phenomenon. Previous investigators had thought that the increase in growth observed with

the decreasing size of the storage container reflected the increase in available oxygen. Zobell showed, however, that the increased growth was the result of organic matter that had been concentrated on the glass.

Despite Zobell's conclusion that the increasing growth was due to the concentration of dilute organics by adsorption to the glass surfaces, some investigators offer evidence that it was solely the presence of solid surfaces that was responsible. Bigger and Nelson (1941) incubated Escherichia coli cells and talc on opposite sides of a dialysis membrane with no resulting increase in the number of cells. When cells and talc were incubated together, an increase was noted. The authors interpret these results to mean that the stimulatory effects of particulates were not due to adhered nutrients. Another possible interpretation of these results is that the nutrients were more than casually associated with the particulate matter.

Button (1967) studied the adsorption of dilute solutions of glucose and thiamine on montmorillonite at pH values of 4, 6.5 and 7. The clay was added to a continuous culture containing a low concentration of one of the nutrients. Since the growth was unaffected by the clay Button concluded that "it seems unlikely that in natural systems the level of suspended organisms using small organic molecules is materially influenced by the level of suspended sedimentary material". Since the adsorption capability of clays is pH dependent, a pH value closer to that found in natural water columns might have given different results.

Rasmussen and Kludt (1970) suggest that particulates are necessary for rapid multiplication in Tetrahymena cultures. They discuss the possibility that nutrient material carried into food vacuoles by the particulates might be responsible for the growth stimulation. However, this possibility was rejected because some non-adsorbing powders also stimulate growth. There are as yet no conclusive experiments proving that particulates cannot function as a nutrient source. This article presents evidence that it is possible for particulates to have a stimulatory effect by their mere presence in the medium.

Particulate material is capable of adsorbing not only nutrients such as polysaccharides (Parfitt and Greenland, 1970) and amino acids (Sieskind and Wey, 1959) but also pesticides (Pfister et al., 1970) and bacterial cells (Hattori, 1970). As much as 99% of the total bacteria in a moderately turbid river are attached to suspended matter (Wuhrman, 1964).

Pfister et al. (1968) have shown that particulates of different densities have stimulatory or inhibitory effects on the growth of certain bacteria. The addition of particulates larger than 0.3 μm to a culture of Streptomyces caused an increase in growth even at concentrations as low as those found in natural waters.

Phosphate buffer (0.3M, pH7) eluted 12 to 36 mg/liter of hexose and from 9 to 25 mg/liter of ammonia nitrogen from river sediments (Hendricks, 1971). The eluted materials were found to increase the respiration rates of enteric organisms by a factor of ten. Since these nutrients could only be eluted by buffers of very high ionic strengths the authors suggested that they would not be readily available for metabolism. It is possible, however, that polluted rivers and lakes would simulate high ionic strength solutions and release some of these bound nutrients. Thus the particulate material represents a potential nutrient source.

In general, most of the studies have been performed on particulate material from sea water. There is a need for information on the composition of particulate material from fresh water and the role that this particulate material plays in the ecology of a natural water system.

MATERIALS AND METHODS

Collection and Concentration of Particulate Material

Five gallon samples were pumped from the Western Basin of Lake Erie at a depth of 15 ft. in Zone A (Pfister et al., 1970). Zone A extends fifteen miles NNW from South Bass Island (Figure 1).

Samples were centrifuged immediately upon return to the laboratory in a Sorvall RC-2B refrigerated centrifuge equipped with a Szent-Gyorgyi and Blum continuous flow attachment at a flow rate of 11 ml/min at 27,000 x g. The collected particulates were resuspended in 50 ml of double distilled demineralized water and frozen at -70 C.

Growth Studies with Particulates

Cultivation of Organisms

The organisms chosen for study were representatives of four of the nine groups of aerobic heterotrophic bacteria found in Lake Erie according to a numerical taxonomic analysis (Kennedy, 1970). A Bacillus, isolate No. 123, and a Pseudomonas, isolate No. 67, were grown in Arginine Glucose Salts (AGS) medium (Appendix A). A Micrococcus, isolate No. 5, and a Flavobacterium, isolate No. 1, were grown in the AGS medium supplemented with growth factors (AGS+ S).

The cultures were transferred monthly on plate count agar (PCA) slants (Difco), incubated at 20 C for three days and then stored at 4 C.

Preparation of Inocula

The Bacillus and the Pseudomonas organisms were grown in 250 ml flasks containing 50 ml of AGS medium on a rotary shaker for 24 hours, then transferred and incubated similarly for 24 hours. The Micrococcus and the Flavobacterium required 72 hours in the supplemented medium to achieve sufficient growth for transfer or study. Suspensions of these organisms were centrifuged at 8,000 x g for 20 min in a Sorvall RC-2B centrifuge and washed three times with sterile double distilled demineralized water. Pellets were diluted with 10 ml sterile double distilled demineralized water and aliquots added to the test flasks or tubes.

Effect of Particle Size on Growth

The particulates were separated into two groups with a 0.45 μm Millipore filter.

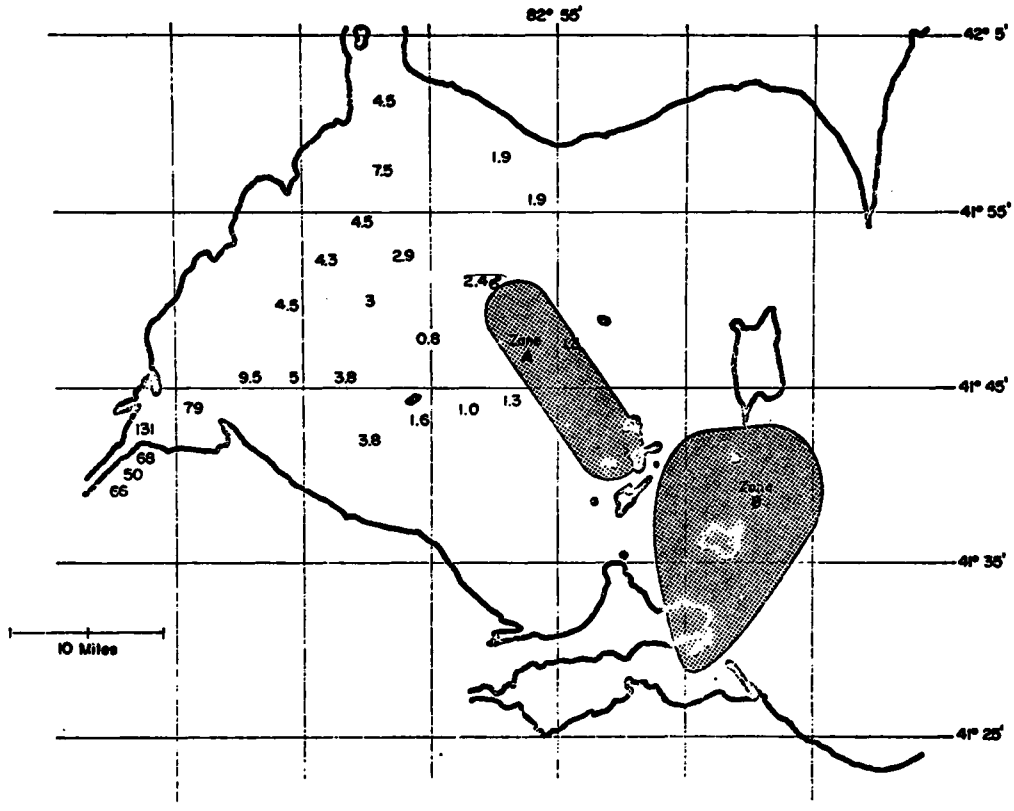

Fig. 1. -- A representation of the Western Basin of Lake Erie showing the sampling area, Zone A

Five ml of the particulates were passed through the filter, and washed twice with 5 ml portions of double distilled demineralized water. The material on the filter was resuspended in 25 ml of double distilled water and added to 25 ml of double strength growth medium. The filtrate plus a glassware rinse, now totaling 25 ml, was added to 25 ml double strength medium. Glucose was omitted so that the differences in the amount of growth observed could be attributed to the particulates. Erhlenmeyer flasks (250 ml) containing 50 ml of medium, were inoculated and incubated for 5 days at 20 C on a rotary shaker. The number of cells were determined during the experiment by plating samples in quadruplicate on PCA.

Particulates as a Source of Carbon or Nitrogen

Particulate material from each of the four seasonal samples was added in 0.5 ml amounts to 4.5 ml medium in screw capped tubes, and then autoclaved. In one series of tubes the glucose was not added (AS), and the particulates substituted as a carbon source. In another series the arginine was not added and the particulates served as source of nitrogen (GS).

One series of controls did not contain glucose, the other did not contain arginine. These controls served to indicate the amount of growth. Medium containing all the nutrients (AGS) was also inoculated as a normal control to indicate the amount of growth the organisms could achieve under these conditions.

Aliquots of prepared inocula were added to the test media in the screw cap test tubes. The tubes were slanted at 19° for greater aeration, shaken on a reciprocating shaker (Lab Line Instruments, Inc.; Chicago, Ill.) for 5 days at 20 C, and sampled to determine the number of cells.

Effect of Detergents on Growth

In a preliminary study, linear alkyl benzene sulfonate (sufonated nalkylene 500, Continental Oil Co., N.Y., N.Y.), Sears biodegradable washing compound (Sears and Roebuck Co., Chicago, Ill.) and Tide washing compound (Proctor and Gamble, Cinn., Ohio) were added to AGS medium at concentrations of 0.3, 3, 30, 300, and 3000 μg/ml. The supplemented medium was inoculated with the Pseudomonas and incubated on a Psychrotherm rotary shaker (New Brunswick Sci. Co., Inc., New Brunswick, New Jersey) at 20 C for three days. The effect of the detergent was observed both visually and microscopically. Since the highest concentration at which growth was observed was 30 μg/ml, this level of detergent containing material was chosen for growth studies of Pseudomonas organisms. One set of flasks, containing each of the three detergents at 30 μg/ml in separate flasks, was inoculated and shaken for 5 days at 20 C. Duplicate flasks of each detergent at each concentration were removed for analysis at zero time, 9 hrs, 44 hrs, 60 hrs and 84 hrs.

The effect of the detergent on growth was determined by estimating deoxyribonucleic acid (DNA) (Clark, 1964). Twenty ml from each flask were centrifuged and the resulting cell pellets were resuspended in 3 ml of double distilled demineralized water in glass tubes. Six ml of the diphenylamine reagent were added and the tubes were heated in a

boiling water bath for 10 min. When cool, the optical density of the solution was determined at 600 nm in a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.). A standard curve was prepared using sperm DNA (Nutritional Biochemicals Corp., Cleveland, Ohio).

Disappearance of the detergent at a concentration of 30 μg/ml was followed by the Methylene Blue method (Standard Methods for the Examination of Water and Wastewater, 1960). Five ml of the zero and 9 hour samples, and ten ml of the 44, 60 and 84 hour samples were diluted to 100 ml with double distilled demineralized water. With phenolphthalein as the indicator, the solution was made alkaline with 1N NaOH, then acidified with 1N H_2SO_4. After transfer to a 250 ml separatory funnel, 10 ml of chloroform and 25 ml of the methylene blue reagent were added. After 30 sec of shaking the chloroform layer was drawn off into a second separatory funnel. The chloroform extraction was repeated three times. The delivery tube of the first funnel was then rinsed with chloroform. The extracts and the rinse were combined in the second funnel. Fifty ml of a wash solution were added to the funnel and shaken for 30 sec. After settling, the chloroform layer was drawn off through glass wool into a 100 ml volumetric flask. The funnel was washed twice and the washes added to the volumetric flask. The solution was diluted with chloroform to the 100 ml mark, and the absorbance determined at 652 nm against a chloroform blank in a Spectronic 20 (Bausch and Lomb, Rochester N.Y.).

Effect of Detergents on Particulate-Microorganism Interactions

The effect of the three detergents on the interaction of the lake particulates and the Pseudomonas organism were studied. The controls consisted of the AS medium plus 0.5 ml of the particulates (P) and AS medium plus each of the detergents (D). The media were prepared as indicated in the table on the following page (Table 1). Glucose was not added to any of the tubes, which were prepared in triplicate, inoculated with the Pseudomonas, and shaken on a reciprocating shaker for 5 days at 20 C. To determine the amount of growth, samples were plated on plate count agar in duplicate and counted after three days of incubation. This experiment was performed three times, once for each detergent.

Analysis of the Particulate Material

Numbers of Aerobic Heterotrophic Bacteria

Water samples were plated on PCA immediately upon collection with a Zobell sampler triggered at 15 ft. Incubation was at 20 C for three days.

Determination of Dry Weight

One ml of the particulate material was added to an acetone cleaned preweighted lyophilization tube. The sample was shell-frozen, evacuated to 60 mm Hg on a lyophilizer and dried for 4 hrs. The vacuum was slowly released (16 hr) and the exterior of the tube was again cleaned with acetone. The tubes were weighed until a constant weight was attained.

Table 1

Ingredients for preparation of medium used in the study of detergent - particulate interactions.

Medium	.5 ml Particulates	30 µm/ml Detergent	AS
P	X[a]		X
D		X	
P + D	X	X	X
WP[b]	X		X
W[b]		X	

a X indicates the ingredients added.

b One and a half ml of the particulates were washed with detergent for ten min on a shaker. The mixture was centrifuged in a Sorvall RC-2B centrifuge for ten min at 27,000 x g. One ml of the supernate (W) was added to the AS. This represented a final concentration of 30 µg/ml detergent. The precipitate (WP), particulates that had been washed with detergent, were added to the AS medium.

Amount of Inorganic Particulate Material

In order to oxidize the organic material, 10 ml of 30% H_2O_2 were added to 5 ml of concentrated particulates. Samples were shaken for one hour. Excess H_2O_2 was removed by slow boiling in a hood. After cooling, the sample was passed through a pre-washed Millipore filter, washed with double distilled demineralized water, dried and weighed to a constant weight.

Free Amino Acids

The free amino acid composition in Lake Erie water was determined by analyzing the supernatant recovered during the centrifugation of the particulate material. Two one-liter samples were analyzed. For the purposes of calculation, one liter of double distilled demineralized water was seeded with 1 ml of the amino acid standards (Beckman, Palo Alto, Calif.). The free amino acids were removed from solution by passage through a ligand exchange column which was prepared by the procedure of Siegel (1967). Chelex 100 (Bio Rad Laboratories, Richmond, Calif.) in the sodium form was mixed three successive times with a saturated solution of calcium chloride. The resin was washed with 25 liters of double distilled demineralized water, rinsed with 1 liter of 3N NaOH, and poured to form a column 10 cm in height. The column was eluted with 1 liter 3N NaOH to remove contaminants added during column preparation. Several rinses with double distilled demineralized water were necessary before the column reached the operating pH of 8. The sample was added at a flow rate of 3 ml/min. The amino acids were eluted with 3N NaOH until the eluent reached pH 10. The eluent, usually less than 5 ml, was evaporated at 40 C on a rotary evaporator. The sample was resuspended in 0.8 ml of pH 2.2 sodium citrate buffer in preparation for amino acid analysis. Two-tenths ml of norleucine was added as an internal standard.

Particulate Amino Acids

One and one half ml of the sample plus one and one half ml concentrated HCl were added to acetone cleaned 5 ml lyophilization tubes. These samples were shell-frozen in liquid nitrogen and attached to a lyophilizer until a pressure of 60 mm Hg was attained. This usually required fifteen min. Because of the very low freezing point of HCl, it was not possible to evacuate more than four tubes at the same time. The tubes were sealed under vacuum and heated at 110 C for the appropriate period of time which varied from 4 to 48 hours. After cooling, the contents of two of the tubes were passed through an HCl rinsed .45 μm Millipore filter. To release the amino acids from the particulates the glass vial and the filter were rinsed three times with 2 ml portions of 6N HCl, followed by two rinses with 3 ml portion of 2N HCl. All of the filtrates were transferred to a round bottomed flask and the suction flask of the Millipore apparatus was washed twice with distilled water. The combined filtrates and suction flask washes were evaporated to dryness in a 40 C water bath with an evaporator (Rinco, Greenville, Ill.) to which a water cooled condensor was attached. A ten ml portion of double distilled demineralized water was added and again evaporated. When a basic hydrolysis was performed, 1.5 ml of 5% NaOH was substituted for HCl.

Amino Acid Analysis

The amino acids from the particulates from the four seasonal samples were analyzed on an amino acid analyzer (Technicon, Tarrytown, N.Y.). For these analyses the prepared samples were suspended in 0.05 ml of water. An internal standard, norleucine (0.15 ml), was added in addition to sufficient 50% sucrose to give a final concentration of 12% sucrose. One half of the sample was added to the column and the other used for a duplicate sample. The time of analysis was approximately 21 hours.

The particulates and the free amino acid samples from May 1971 were analyzed on a Model 116 Amino Acid Analyzer (Beckman, Palo Alto, Calif.). For these analyses the samples were suspended in 0.8 ml of a pH 2.2 sodium citrate buffer. As an internal standard, 0.2 ml of norleucine was added. Between 0.35 and 0.4 ml of the sample was added to each column of the analyzer. The time of analysis was one hour on the basic column and 3.5 hrs on the acidic and neutrals column.

Calculation of the Amount of Amino Acid

The amount of amino acid was recorded using a colorimetric reaction with ninhydrin at 440 and 570 nm. The area under a given peak is an indication of the amount of amino acid present. When considered as a rectangle, it is necessary to determine the height, and the width at half-height. The height is determined by subtracting the baseline optical density reading from the highest optical density attained. Since the chart prints one symbol every 6 seconds, the number of symbols estimated to the nearest tenth above the half height line equals the width of the peak. The height times the width equals the area.

Addition of an internal standard provided a check to compensate for variations in the ninhydrin, and other variables such as the flow rate. To use this information, one calculates a norleucine equivalent (NLE).

$$\text{N.L.E.} = \frac{\text{area of norleucine in standard}}{\text{area of amino acid in standard}}$$

The amount of the amino acid in the sample chromatogram is compared to the amount in the standard by incorporating the NLE into the basic equation:

$$\mu\text{M} = \text{N.L.E.} \times \frac{\text{area amino acid in sample}}{\text{area norleucine in sample}}$$

The value obtained indicates the μM content of the amount put on the column. Further calculations must be made to determine the amount of amino acid in the original sample.

RESULTS

Effect of Particle Size on Growth

The effect of particulates larger than 0.45 μm and smaller than 0.45 μm on the growth of four Lake Erie isolates was investigated. All of the particulates were stimulatory for the Pseudomonas organism, when substituted for glucose, with the smaller particulates the most effective (Figure 2). The greatest increase in cell numbers was noted for the Micrococcus, with the smaller particles again the most effective. The reverse was observed with the Bacillus. The smaller particulates supported the same number of organisms as the unfiltered particles, while the larger particulates stimulated an increase in growth. Microscopic observations revealed that the Bacillus was able to sporulate, using the particulates as a carbon source. This is an indication of the presence of glutamic acid which is a requirement for sporulation in many Bacillus species. However, the spores did not appear to germinate upon heat-shock treatment indicating either faulty spore formation, or the lack of a necessary nutrient, such as an amino acid, for germination. Some Bacillus species require alanine or other amino acids for germination to occur. The smaller particulates were also stimulatory for the Flavobacterium, while the larger particulates inhibited their growth. This inhibition by the larger particulates appeared to be neutralized by the smaller particulates (Figure 2, Col. D).

In general, both sizes of particulates supported growth, with the smaller particulates the more effective. The only exception was the inhibitory effect of the larger particulates on the Flavobacterium. In no case did the effect of these two groups of particulates appear to be additive (Col. D). In other words, the growth in Col. D does not exceed the amount achieved with either size separately. Except in the situation of the Flavobacterium, the unfiltered particulates were either equal to or less than the lower level of growth recorded.

Although there was a large increase in the actual number of organisms, only the Micrococcus consistently underwent more than one division cycle (Table 2). Since the Micrococcus are smaller than any of the other organisms tested, they should be capable of initiating a greater number of divisions for the same nutrient supply. There does not appear to be any correlation between the number of generations which occurred with either of the sized groups of particulates and the number of generations which occurred with the unfiltered particulates. The increase in the number of cells was determined for each sample by subtracting the number of cells in the control (AS) from the number of cells in each sample. The number of generations represented by this increase in cell numbers was determined by successive doublings of the control population and subtraction of this number from the noted increase in cell numbers. Each complete subtraction was considered to represent one generation.

Effect of Surfactants on Growth

Five concentrations (0.3, 3, 30, 300, and 3000 ppm) of Sears' biodegradable laundry compound(s), Tide (T) and linear alkyl benzene sulfate (LAS) were tested on the growth of the Pseudomonas organism. No growth was apparent in the two highest concentrations of detergent compound. Microscopic observations revealed extreme amounts of cellular debris.

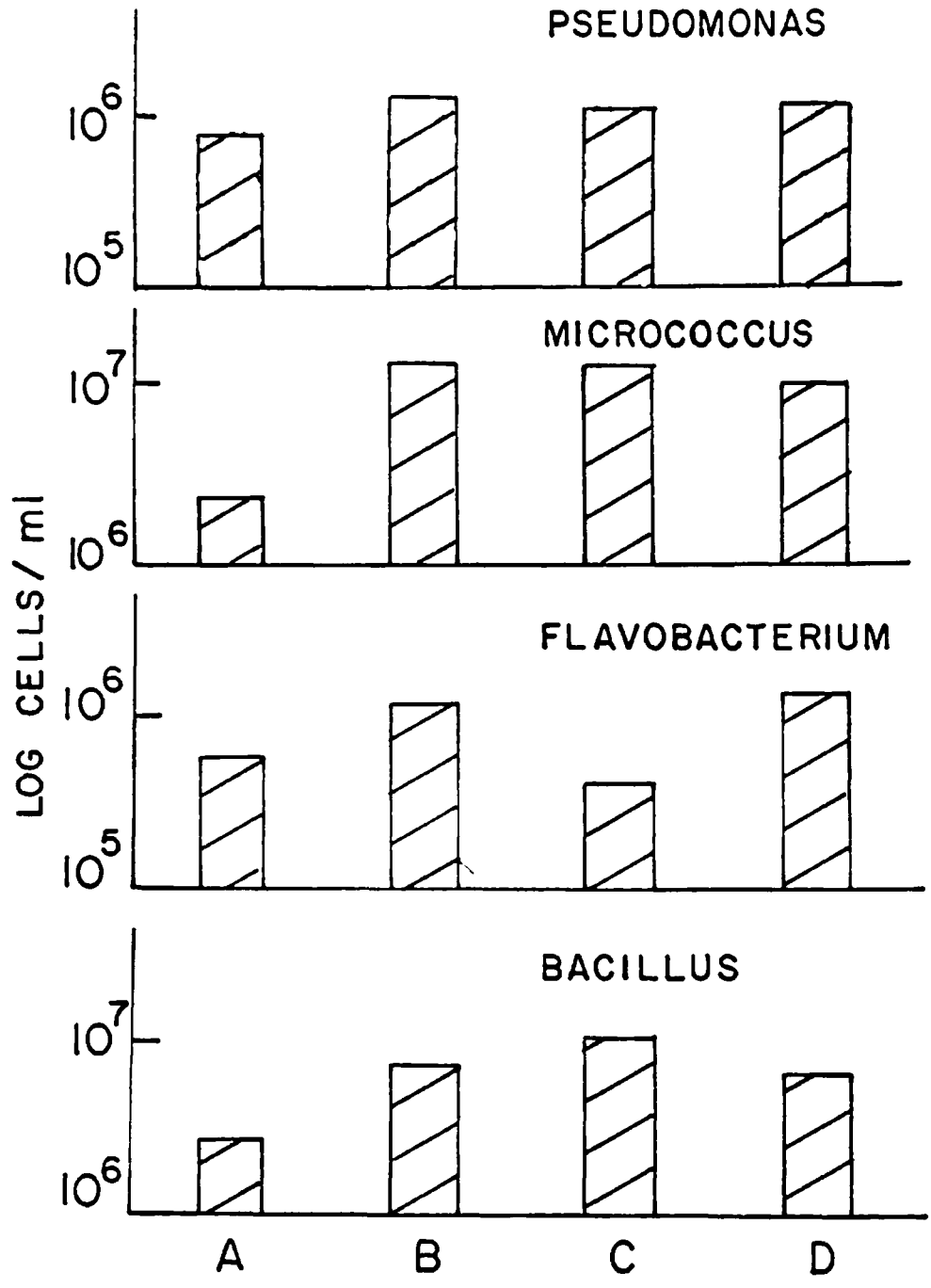

Fig. 2. -- The effects of particulates of different sizes, collected on March 24, 1970, on the growth of four Lake Erie bacteria. Column A represents the number of cells/ml in the control, which did not contain glucose. Column B represents the number of cells/ ml with the particulates smaller than 0. 45μm. Column C represents the number of cells/ml with the particulates larger than 0. 45 μm. The particulates in Column D were not filtered, and represent the number of cells/ml with both sizes of particulates combined.

Table 2

The increase in the number of organisms noted with the particulates smaller than 0.45 μm, larger than 0.45 μm and the combined or unfiltered particulates. These numbers represent the increase in growth above the control. The number of generations that this increase in cell numbers represents is also given.

	< 0.45 μm	> 0.45 μm	Unfiltered
Micrococcus			
Increase in Numbers	1.35×10^7	1.0×10^7	8.5×10^6
Number of Generations	2.3	2.0	1.8
Flavobacterium			
Increase in Numbers	5.6×10^5	- - - - -	7.1×10^5
Number of Generations	0.8	- - - - -	1.0
Bacillus			
Increase in Numbers	5.0×10^6	8.0×10^6	4.2×10^6
Number of Generations	0.6	2.6	0.4
Pseudomonas			
Increase in Numbers	7.0×10^5	3.5×10^5	3.5×10^5
Number of Generations	0.87	0.4	0.4

In 3 and 30 μg/ml detergent, an increase in growth above the control was observed. Moreover, the green water soluble pigment observed with some Pseudomonas was formed while in AGS medium the pigment did not form. The Pseudomonas organism normally clumped during the logarithmic and stationary phases of growth but in the presence of .3, 3 and 30 μg/ml detergent, the clumps assumed the shape of snow-flakes or stars (Figure 5). Microscopic observations revealed that these flakes were composed of small bacillary shaped organisms. During the centrifugation for the determination of DNA large amounts of a white viscous polymer were observed in the cell samples treated with detergents. There did not appear to be an increase in the number of cells with the 0.3 μg detergent/ml.

The growth of the organism was examined over a period of 84 hours. In the presence of 1 g/liter glucose and 30 μg detergent/ml, there was an increase in cell DNA content over the control (Figure 6). A control tube of sonified cells showed similar amounts of DNA/ml indicating that the diphenylamine reagent disrupted the cells sufficiently to permit the analysis without pre-treatment of cells. The magnitude of the increase was very similar for all three detergent compounds with the LAS the most effective and the Tide the least. The concentrations of detergent remaining at 84 hours was determined. Detergent residue from LAS and Tide was not detectable by the Methylene Blue determination at that time because there is always a low residual level of methylene blue reactive matter and the test is not valid at low concentrations. The Sears detergent did not react with methylene blue and could not be measured. This indicated that the Sears is a different type of detergent than the linear alkyl benzene sulfonate or alkyl benzene sulfonate type.

Interactions of Detergents and Particulates

To study the interactions of surfactants and particulate material, 30 μg/ml of each of the three detergent compounds were added to the medium containing particulate material which was collected on May 19, 1971 (Figure 7). The surfactant may physically alter the particulate material or may cause the release of substances or microorganisms into the surrounding medium. To discern the possible mode of action, the particulates were briefly exposed to the surfactant and then separated by centrifugation. Both the particulates and the wash were then incubated separately.

In the particulate medium (P), the Pseudomonas exhibited the expected increase in growth above the control. The Tide and LAS detergents (D), in the absence of glucose, did not cause large amounts of growth in contrast to the data shown in Figure 6.

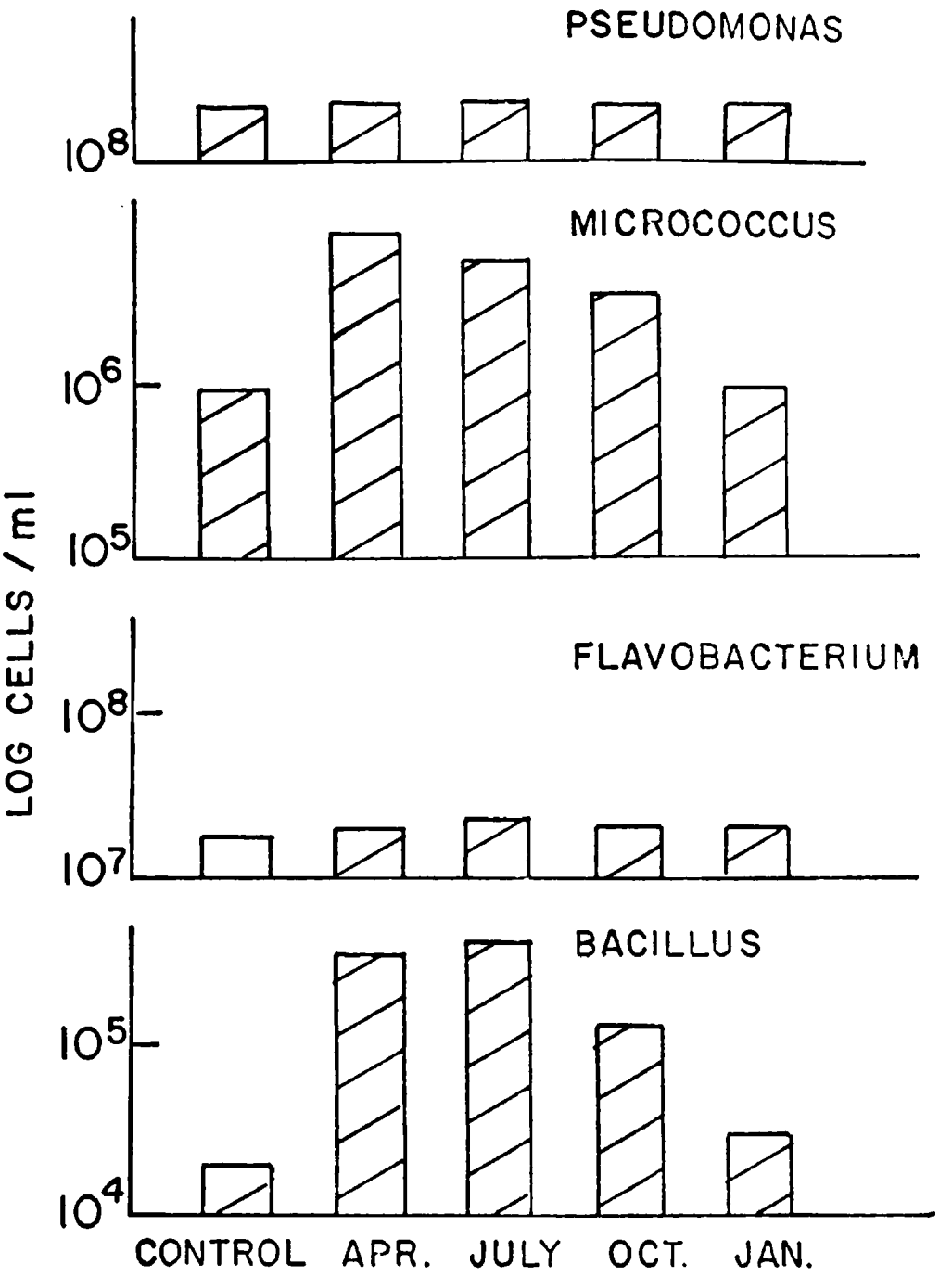

Fig. 3. -- The particulates from the April, July and October, 1970 and the January, 1971 samples of Lake Erie water as a source of carbon for four Lake Erie isolates, a Micrococcous sp., a Pseudomonas sp., a Flavobacterium sp., and a Bacillus sp. There was no carbon source in the control.

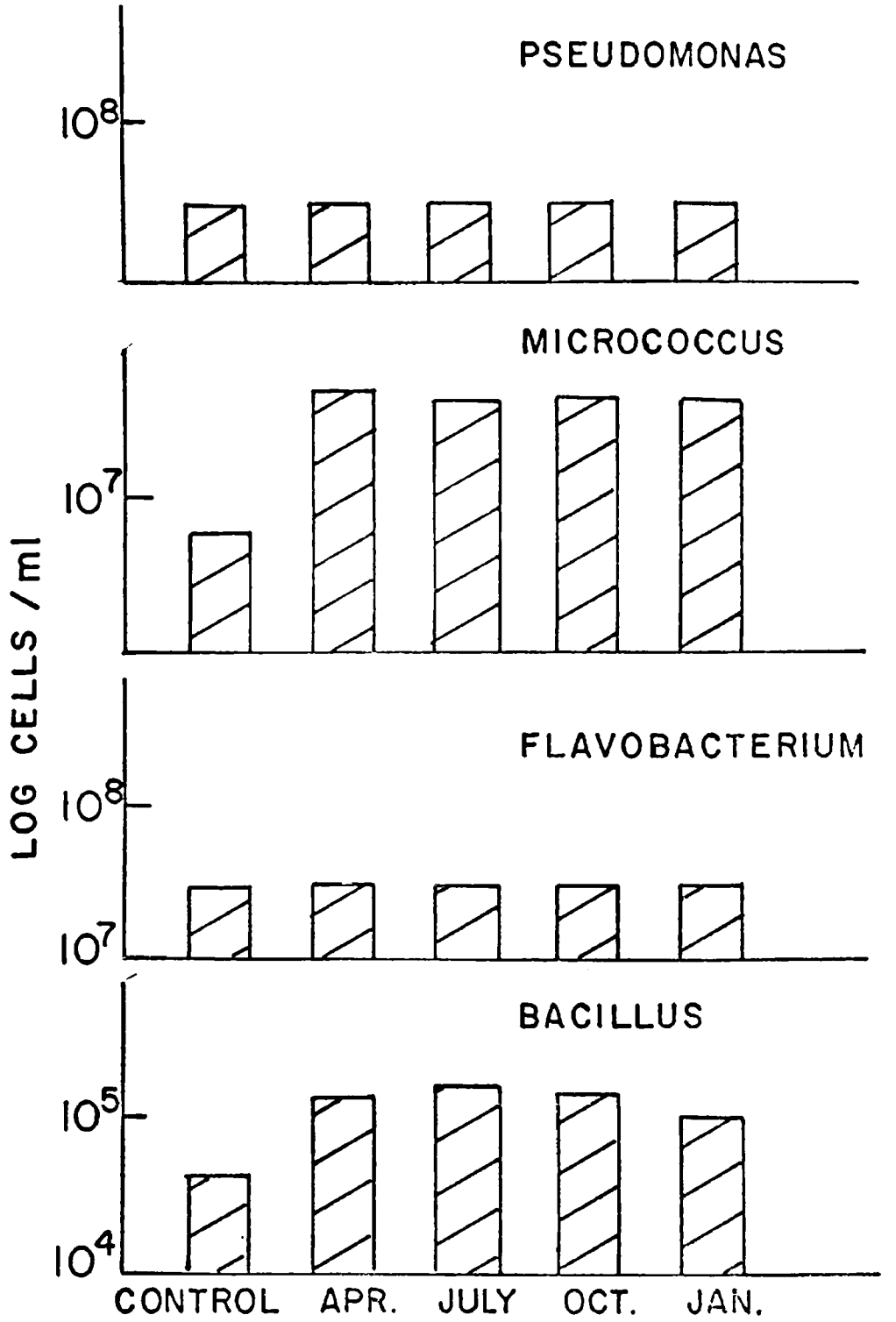

Fig. 4. -- The particulates from the April, July and October, 1970 and the January, 1971 samples of Lake Erie water as a source of nitrogen for four Lake Erie isolates, a Micrococcus sp., a Pseudomonas sp., a Flavobacterium sp., and a Bacillus sp. There was no nitrogen source in the control.

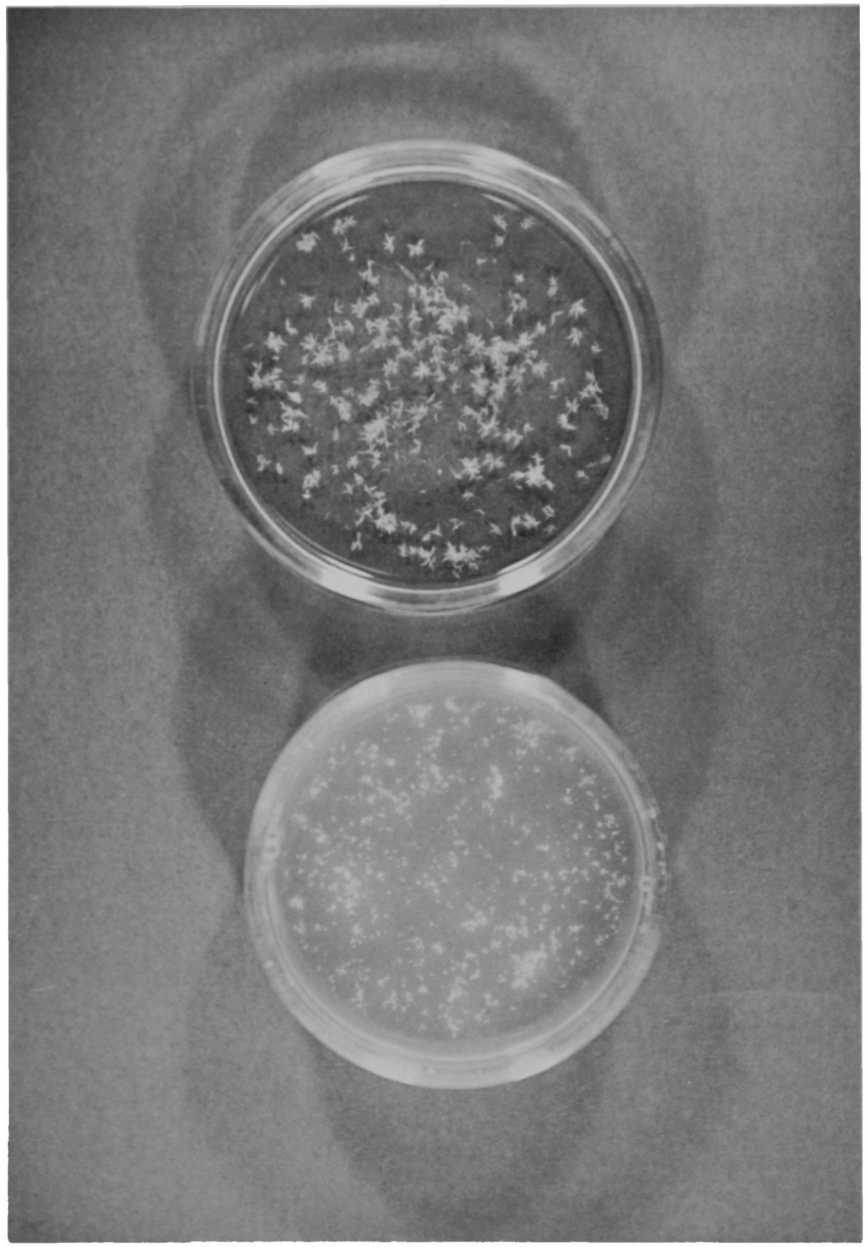

Fig. 5. -- Photograph of Pseudomonas cells in control AGS medium (upper) showing normal cells clumping, and cells in AGS medium plus 30 μg/ml detergent (lower) showing flake-like stars.

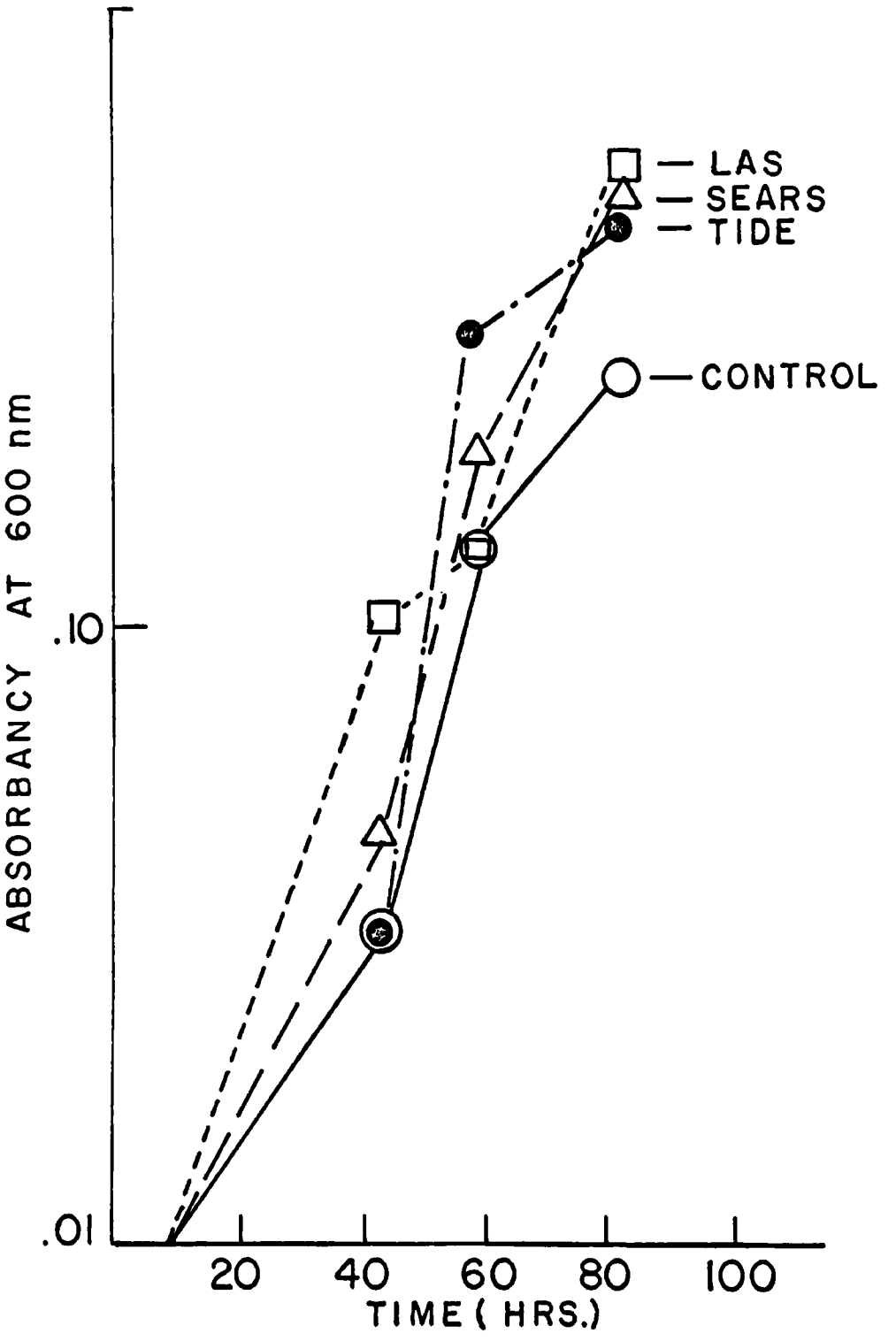

Fig. 6. -- Growth curve as determined by DNA analysis of Pseudomonas organism in AGS medium plus 30 μg/ml LAS (dotted line), 30 μg/ml S (solid line) and 3- μg/ml T (broken line). The lower solid line was the control grown in AGS medium.

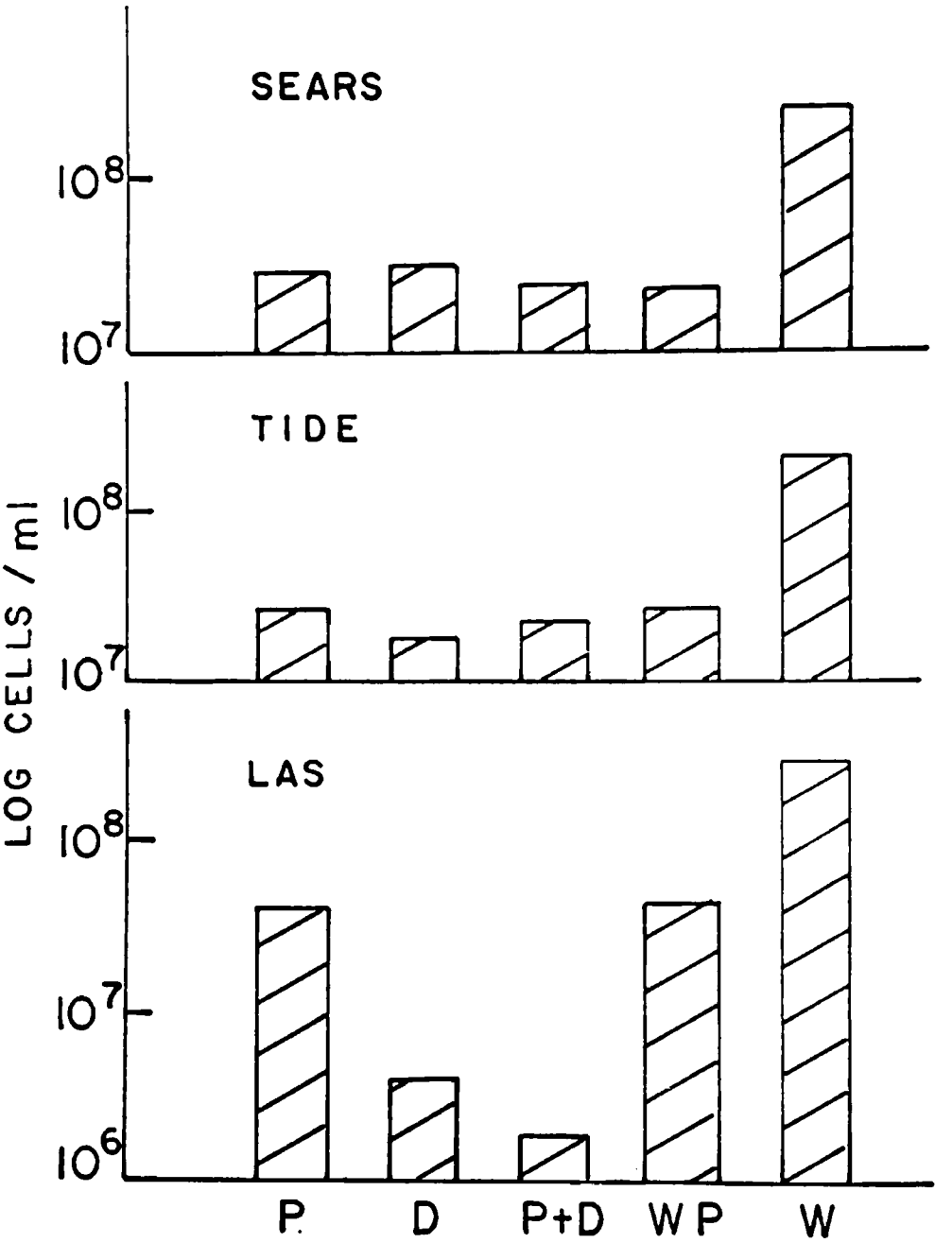

Fig. 7. -- The effect of detergent compounds on the particulate material from May, 1971, as a source of carbon for a Pseudomonas orgamism isolated from Lake Erie.

P 0.5 ml particulate material (control)

D 30 μg/ml detergent

P + D 0.5 ml particulates plus 30 μg/ml detergent

WP detergent rinse

DISCUSSION

This research was undertaken to investigate the role of the particulate material in Lake Erie water. One of the possible functions of particulate material, its ability to serve as a nutrient source for bacteria, was investigated. The results suggest that the particulates larger than 0.45 μm and smaller than 0.45 μm have different effects on growth (Figure 2). Since the particulates smaller than bacteria were generally more stimulatory than the larger particles, we can conclude that these two size groups are different in composition. This difference may be the result of the association of different organic compounds or just as unequal association of the same organics with both particulates. A third possibility is that the difference may be due simply to a greater number of the small particulates.

The question arises as to why the effects of these two groups were not additive since one would expect that an increase in available organics would cause a corresponding increase in growth. One possible explanation for this is that the particulates may have different effects when in association with each other. The smaller particles may physically block available sites or nutrients on the larger particulates. This explanation may also account for the inhibition of the Flavobacterium by the larger forms, in that the inhibitory substances (possibly pesticides) may be blocked or neutralized in the presence of the smaller particulates.

It can be concluded that the particulate material from Lake Erie can serve as a source of carbon and nitrogen for organisms isolated from the lake (Figure 3, Figure 4). The samples from January were poor carbon sources. Whether this was the cause or the result of the low level of growth in the lake is not known since it was not possible to measure the dry weight of this sample with the volume available. However, the sample was visibly less turbid than the other seasonal samples.

An attempt was made to determine the amount of carbohydrate material in the particulates by paper chromatography. The concentration necessary to give positive results was greater than the available. However, the control, a mixture of twelve carbohydrates, was a successfully chromatographed. These results indicate two possibilities: One, that the carbohydrate material is bound too tightly; the other, that there is little carbohydrate material available in Lake Erie. The latter is the more likely alternative. The results suggest that the carbohydrate concentration in the particulates is less than 10 μg/liter. Handa and Tominaga (1969) found that 100 liter water samples were necessary for carbohydrate analyses.

The ability of these particulates to adsorb ions and molecules means that the standard methods of analysis for carbon and nitrogen cannot be used. When the Lowry, Anthrone and a modified Kjeldahl analyses were attempted, the particulates adsorbed all of the resulting color. It might be possible to elute or distill the products but the possibility of obtaining accurate quantitative results are low.

All of the four seasonal samples contained sufficient nitrogen to support growth (Figure 4), and the amount of growth was relatively the same. This suggests either that there is a nutrient other than nitrogen limiting the growth or that there is a constant amount of

Table 3

An analysis of the particulate material in four seasonal Lake Erie samples, showing cells/ml, µg amino acid/ml, dry weight and percent inorganic composition of the particulate material.

	APRIL	JULY	OCTOBER	JANUARY
CELLS/ml*	9840	15480	12280	8600
µg Amino Acid/ml	0.1	0.7	0.1	0.02
Dry Weight - µg/ml	2.6	4.5	3.8	----
% Inorganic	----	72	----	----

* Numbers of bacterial cells/ml were obtained on the uncentrifuged lake water.

nitrogen available throughout the year. The main sources of nitrogen are amino acids, complex organics, amino sugars, ammonia compounds and nitrates. The question arises as to which of these was responsible for the increased growth. The inability of these particulates to bind nitrates has already been mentioned. Although complex organics are a possible source of nitrogen, most of the research on marine particulates has indicated that amino acid nitrogen is the predominant form. The amino acid composition has been investigated and it was found to vary from 0.2 to 6.9 μg amino acid/ml (Table 4). This amount was more than sufficient to provide for the increase in cells noted (Table 2). The final possibility is that there is available a constant amount of ammonia. Evidence from the modified Kjeldahl analysis indicates that the particulates bind ammonia very strongly. The answer must fulfill the requirement of a low constant level of available nitrogen whether from ammonia, amino acids or through some regulatory mechanism. Since the microorganisms have different nutrient requirements, different sources may supply them.

The aerobic heterotrophic bacteria probably make a far greater contribution metabolically than as sources of nutrients. The 15,480 cell/ml found during the month of July contribute only 1.5×10^{-2} μg/ml to the dry weight. This represents less than 1% of the total dry weight of the particulate material. Seki (1970) calculated that the microbial population represented about 2% of the organic particulate material. With the estimation that 60% of the dry weight of the bacterial cell is carbon, we can calculate that there was approximately 9×10^{-3} μgC/ml of bacterial biomass in the particulate material from Lake Erie. If this figure represents 2% of the organic particulater material as Seki calculated, there would be 0.45 μg C/ml of organic particulate material in the Lake Erie particulates. In the July sample the dry weight of the particulate material was 4.5 μg/ml. Therefore, it is possible to make a rough calculation that the particulate organic material in Lake Erie forms 10% of the total particulate material.

Another series of experiments investigated the interactions of detergent compounds and the particulate material from Lake Eire on the growth of the Pseudomonas organism. When the Pseudomonas was tested against five concentrations of each detergent, the 300 and 3000 μg/ml concentrations lysed the cells. The 30 μg/ml quantity was then chosen as the optimal concentration. In the standard shake flask method for determination of percent of biodegradability, the normal concentration of detergent is 20-30 μg/ml.

The Pseudomonas organism was able to utilize all three of the detergents as evidenced by the increase in growth above the control (Figure 6). The differences in growth among the three detergents may be the result of the surfactant or one of the additives in the Sears or Tide sample.

The effect of the detergent on the clumping of the cells was very striking, as was the production of a green water soluble pigment. As noted in the results, the AGS grown Pseudomonas does not appear to have a polymer matrix surrounding the cell. However, when grown in the detergent, a white, viscous polymer was noted upon centrifugation of the cells. One might expect that this was due to excess carbon. However, the cells are normally grown on 1000 μg/ml glucose so the addition of 30 μg/ml detergent is probably not a significant increase in carbon.

Table 4

Results from 24 hour acidic hydrolysates of four seasonal Lake Erie particulate samples. Recorded as µg amino acid/10 liters.

AMINO ACID	APRIL	JULY	OCTOBER	JANUARY
Aspartic Acid	127	1031	119	---
Threonine	84	505	70	---
Serine	141	437	85	---
Proline	---	81	---	---
Glycine	112	616	79	---
Alanine	104	783	10	---
Valine	71	584	96	211
Methionine	---	12	---	---
Isoleucine	54	338	121	18
Leucine	87	551	101	28
Tyrosine	31	317	---	145
Phenylalanine	54	373	64	17
Lysine	64	615	111	---
Histidine	32	173	---	---
Arginine	37	569	81	---
Glutamic Acid	+	+	+	---
TOTAL	999	6985	1025	227

Table 5

Comparison of the particulate and free amino acids in a sample of Lake Erie water. The particulate amino acids are represented as µg amino acid/10 liters. Free amino acids are marked + if present, and --- if absent.

AMINO ACID	SUPERNATANT	PARTICULATE
Lysine	---	280
Histidine	+	60
Arginine	---	210
Aspartic Acid	+	480
Threonine	+	580
Serine	+	700
Glutamic Acid	+	550
Proline	---	240
Glycine	+	780
Alanine	+	440
Valine	+	400
Methionine	+	70
Isoleucine	+	257
Leucine	+	330
Tyrosine	---	100
Phenylalanine	---	157
Cysteine	---	+

It would be interesting to investigate the production of the polymer and the effects of detergents on floc-forming organisms. Floc-formation is important in many systems such as water treatment plants, trickling filters and the uptake of ions and pesticides in natural waters.

Interfaces, whether air-water, cell-surface to water, particle to cell or particle to water are important in that they are the site of concentration for organic compounds, ions and surface-active agents. The effect of detergents on microbial-particulate interactions was studied (Figure 7). In the control tubes of particulates (P) there was the anticipated increase in growth over the control. However, in the tube containing detergent plus a medium (D) there was little growth except in the case of Sears. In the growth curve (Figure 6), where glucose had been added, a large increase in growth was noted. The enzyme system for detergent utilization appears to be an inducible system (Van Marion, 1966), and it is probable that the Pseudomonas was not able to synthesize the indicible enzyme system without a carbon source present.

When the particulates and detergent were incubated together (Figure 7, P + D), the results were surprising. The organisms incubated with the LAS and the particulates did not appear to grow at all. There was some growth in the Sears and Tide but not so much as was achieved with the particles alone. It was thought that the detergents would reduce the surface tension and release more nutrients into the environment for the bacteria. It is also possible that the detergents interfere with the particulate/bacterial association.

Weber and Coble (1968) have shown that pesticides are tightly bound to clays. It is possible that the detergents released an inhibitory substance, such as one of the pesticides. The destructive effect of high concentrations of detergent on the bacterial cell membrane would only be increased by the release of such a substance. Little work has been done on the combined effect of pollutants. Dugan (1967) showed that goldfish exposed to alkyl benzene sulfonate (a detergent) were more susceptible to the toxic effect of DDT and dieldrin. The interactions of pollutants such as detergents and pesticides are an important area since there is a constantly increasing input of pollutants into the environment.

In general, the detergents reduced the amount of growth when in prolonged contact with the Lake Erie particulates. In an attempt to elucidate the mode of action of the detergent, the particulates were exposed to detergent for 10 min. The results of this experiment indicate that there is a difference between long and short term exposure to detergents. When the particulates were exposed to detergents for a short period of time there was no difference in the amount of growth supported as compared to the particulates alone. However, the detergent rinse supported a great deal of growth. It is possible that the detergent had caused release of some normally unavailable nutrients. These nutrients might then enable the organism to synthesize the inducible enzyme system and utilize the detergent, resulting in a great increase in growth.

The July sample contained the largest amount of particulate amino acid. One would expect the large concentration of amino acids and other nutrients during the summer months because of the great increase in the number of organisms in the lake. Results of the amino acid analyses of the particulates probably reflects the amino acid composition of the

dominant mircobial species at the time of sampling.

Siegel (1967) compared the particulate and free amino acid content of water collected from Buzzards Bay near New Bedford, Conn. He found 175 μg of particulate amino acid/liter. Analysis of the Lake Erie particulates range from 22 to 698 μg amino acid/liter. Siegel found arginine, lysine, and glutamic acid to be the predominant forms at the time of his analysis. The predominant amino acids in the lake particulates vary with the time of sampling. However, aspartic acid, serine, glycine were found in high concentrations in most of the samples tested. Serine and glycine were the predominant forms in the Buzzards Bay sample while serine and glutamic acid were the most prevalent in Lake Erie. The amount of the particulate matter for both seawater and freshwater were in the same range; the values for the free amino acids were higher in the ocean.

An attempt was made to correlate the presence of an amino acid in its free form with its solubility in water in water as given in the Handbook of Chemistry and Physics (1959). However, there did not appear to be any relationship. At least a gram of most of the amino acids was soluble and proline, which was not found in the free form at all, was almost infinitely soluble. It was interesting to note that serine and glycine were quite prevalent in both the free and bound forms. Although the free amino acid data was not quantitative, it did establish a concentration of at least 10 μg/ml free amino acid.

Experimentation in the area of ecological relationships presents many problems. The variance in parameters and the need to duplicate environmental conditions are examples. The study of particulate material is no exception in this regard, and suffers further from a lack of definition. A glance at the literature shows that one author considers only plankton as "true" particulates, another only clays. The variety of ways in which the particulates are separated for study causes enormous variance before experimentation has even begun. In the research discussed in this dissertation, anything in suspension, from 0.2 μm (colloidal size) to several micrometers, has been considered as particulate material. This definition still begs the question for there are some particles of very large dimensions that can be found suspended in water.

Additional problems have arisen with the methods of experimentation used to detect the influence of particulate matter. Waksman and Carey (1935) found little difference in the amount of growth in filtered and unfiltered seawater. Interpretation of these results can lead to the conclusion that the contribution of the particulate material is inconsequential. Comparisons of growth in the presence of particulates to growth in seawater are not valid. Seawater contains non-particulate nutrients in the order of 5 to 6 mg/liter (Harvey, 1957) and in a closed system there will be excessive amounts of growth (Zobell, 1943). However, in an open system this material is not sufficiently concentrated to be available for growth and here particulate material may contribute. Heukelekian and Heller (1940) showed that E. coli cells would not grow at concentrations of nutrients less than 2.5 mg/liter, unless particulate material was present. When the nutrient concentration exceeded 25 mg/liter, the E. coli culture was unaffected by the presence or absence of solids.

Experiments must be designed to allow for the small nutrient effect provided by these particulates. Inocula should not exceed values of 10^3 cells/ml in distilled water, and 10^5

cells/ml in specially cleaned organic free water. When the inoculum is too large the increase in the numbers of cells due to the particulates cannot be measured. An example of this can be seen in Figure 2. In most of the experiments performed the inoculum was in the range of 10^3 cells/ml. However, the resulting growth in carbon-free AGS medium reached 10^6 cells/ml. It has been found by other investigators that E. coli can grow in basal salts medium prepared with distilled water and reach a final population of 10^6 cells/ml (Postgate and Hunter, 1962). Shetata and Marr (1971) developed an involved cleaning procedure that did not permit an increase in number of cells.

Some of the other problems encountered in this area of research are enumeration of organisms, interpretation of results, and storage of the water samples. The traditional method of adsorption spectrophotometry cannot be used because of the particulates. Since bacteria and other organisms comprise a portion of the particulates, measurement of DNA is also misleading. Many of the bacteria attach to the surfaces of particulates making plate counts inaccurate. The latter method was chosen for this work because the error would be in underestimation of the amount of growth. One of the difficulties in analyzing the amino acids in Lake Erie was the necessity of returning to the laboratory and storing the water. However, Baker (1968) showed that there is relatively little loss of organic materials upon prolonged storage at -19 C. Recovery was essentially complete after 20 days and from 82 to 92% was recovered after 96 days.

Appendix A. Media Components

Table 6

Chemical composition of the Arginine Glucose Salts (AGS) medium.

	g/liter
Glucose	1.0
Arginine Hydrochloride	1.0
$MgSO_4$ $7H_2O$	0.4
K_2HPO_4	4.0
KH_2PO_4	2.0

Double distilled demineralized water was added to a one liter volume. The final pH of the medium was 7.2 after autoclaving.

Table 7

Chemical Composition of the Supplemented

Arginine Glucose Salts Medium.

	g/liter
Glucose	1.0
Arginine Hydrochloride	1.0
$MgSO_4 \cdot 7H_2O$	0.4
K_2HPO_4	4.0
KH_2PO_4	2.0
Pyridoxine	0.01
Xanthine	5×10^{-3}
Guanine	5×10^{-3}
Adenine	5×10^{-3}
Uracil	5×10^{-3}
Riboflavin	5×10^{-3}
Para Amino Benzoic Acid	5×10^{-3}
Beta Alanine	2.5×10^{-2}
Nicotinic Acid	2.5×10^{-2}
Biotin	1×10^{-4}
Folic Acid	5×10^{-5}
Vitamin B_{12}	3×10^{-6}

Double distilled demineralized water was added to a one liter volume. The final pH of the medium was 7.2 after autoclaving.

Appendix B. Standard Curve

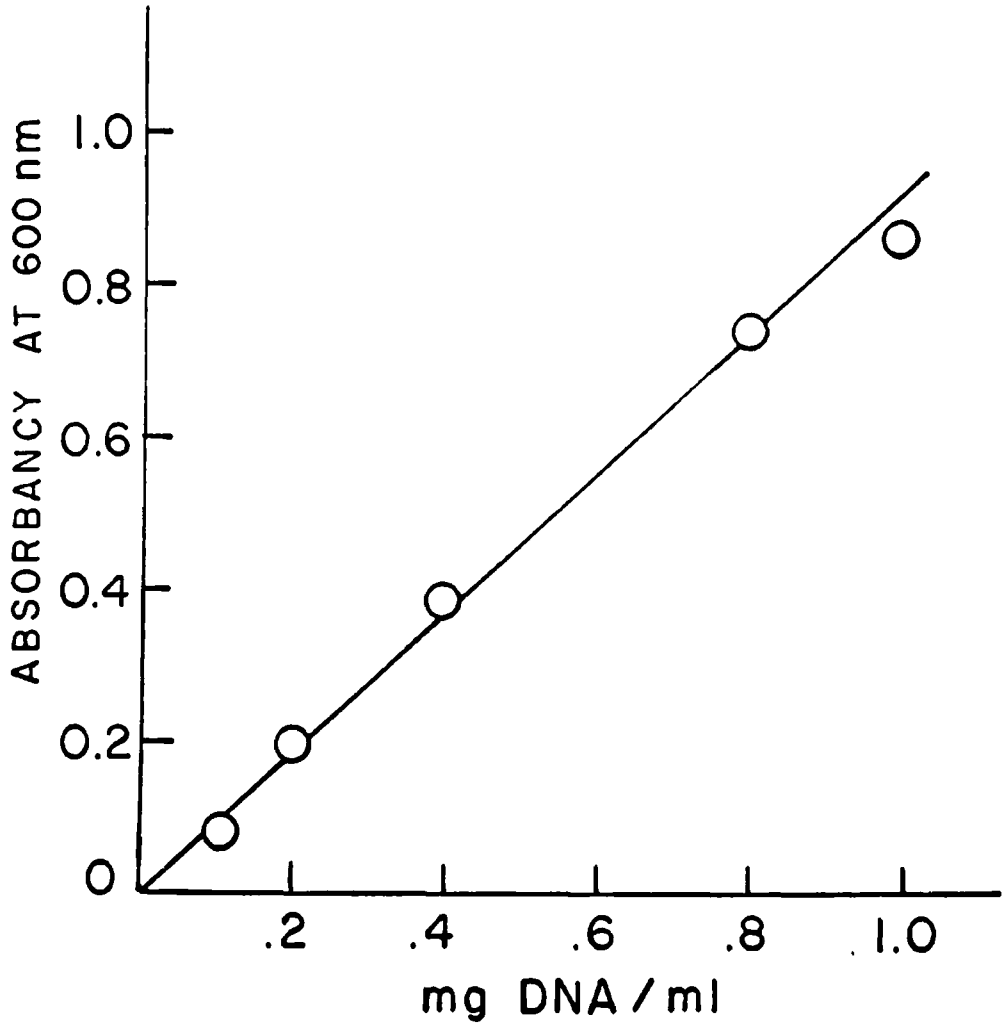

Fig. 8. -- Standard curve used for DNA quantitative.

BIBLIOGRAPHY

Baker, R.A. 1968. Freeze concentration of microorganics in water. In Microorganic matter in water. American Society for Testing and Materials. Philadelphia, Pa. pp. 65-77.

Bigger, J.W., and J.H. Nelson. 1941. The growth of coliform bacilli in distilled water. J. Pathol. Bacteriol. 53: 189-206.

Brock, T.D. 1966. Principles of microbial ecology. Prentice-Hall Inc., Englewood Cliffs, N.J. pp. 18-22.

Button, D.K. 1967. Effect of clay on the availability of dilute organic nutrients to steady-state heterotrophic populations. Limnol. and Oceanog. 14: 95-100.

Clark, J.M., Jr., (ed.) 1964. Experimental Biochemistry. W.H. Freeman and Company, San Francisco. P. 135.

Dugan, P.R. 1967. Influence of chronic exposure to anionic detergents on toxicity of pesticides to goldfish. J. Water Poll. Control Fed. 39: 63-71.

Duursma, E.K. 1960. Dissolved organic carbon, nitrogen and phosphorous in the sea. Ph.D. Thesis, J.B. Wolters, Groningen.

Fox, D.L. Oppenheimer, C.H. and J.S. Kittredge. 1953. Microfiltration in oceanographic research. J. Mar. Res. 12: 233-243.

Handa, N. and H. Tominaga. 1969. A detailed analysis of carbohydrates in marine particulate matter. Internat. J. on Life in Oceans and Coastal Waters 2: 228-235.

Gordon, D.C., Jr. 1970. A microscopic study of organic particles in the North Atlantic Ocean. Deep Sea Res. 17: 175-185.

Harvey, H.W. 1957. The Chemistry and fertility of seawater, second edition. Cambridge University Press, London, England.

Hattori, T. 1970. Adhesion between cells of E. coli and clay particles. J. Gen. Appl. Microbiol. 16: 351-359.

Hendricks, C.W. 1971. Enteric bacterial metabolism of stream sediment eluates. Can. J. Microbiol. 17: 551-556.

Heukelekian, H. and A. Heller. 1940. Relation between food concentration and surface for bacterial growth. J. Bacteriol. 40: 547-558.

Holm-Hansen, O. 1969, Determination of microbial biomass in ocean profiles. Limnol. and Oceanog. 11: 740-747.

Jorgensen, C.B. 1961. The food of filter feeding organisms. Symp. on zoological production. Cons. Perm. Int. Expl. Mer. Copenhagen.

Kennedy, R.S. 1970. Cytological and physiological effects of chlorinated hydrocarbon pesticides and dissolved oxygen concentration on a Pseudomonas sp. isolated from Lake Erie. Ph.D. Thesis. The Ohio State University.

Krogh, A. 1934. Conditions of life at great depths in the ocean. Ecol. Monog. 4: 430-439.

Krogh, A. and Lange, E. 1932. Quantitative Untersuchungen uber plankton, kolloide und geloste organische und anorganische substanzen in dem furesee. Int. Rev. Hydrobiol. 26: 20-53. In E. Hutchinson. 1957. A treatise on limnology. John Wiley and Sons, Inc., New York.

Lee, G.F. 1970. Factors affecting the transfer of materials between water and sediments. Univ. of Wisconsin, Eutrophication Info. Program. p. 37.

Lisitzen, A.P. 1959. Suspended matter in the ocean. Preprint Int. Oceanog. Congress, N.Y. pp. 470-471.

Manheim, F.T., R.H. Meade and G.C. Bond. 1970. Suspended matter in surface waters of the Atlantic Continental margin from Cape Cod to the Florida Keys. 167: 371-376.

McLaren, A.D. 1963. Biochemistry and soil science. 141: 1141-1147.

Parfitt, R.L. and D.J. Greenland. 1970. Adsorption of polysaccharides by montmorillonite. Soil Sci. Amer. Proc. 34: 863-865.

Parsons, T.R. 1963. Suspended organic matter in sea-water. In M. Sears (ed.) Progress in Oceanography. Pergamon Press, London. pp. 205-239.

Parsons, T.R. and J.D. Strickland. 1962. On the production of particulate carbon by heterotrophic process in sea water. Deep Sea Res. 8: 211-222.

Peterson, W.H., F.B. Fred and B.P. Domogalla. 1925. The occurrence of amino acids and other organic nitrogen compounds in the lake water. J. Biol. Chem. 63: 287.

Pfister, R.M., P.R. Dugan and J.I. Frea. 1968. Particulate fractions in water and the relationship to aquatic microflora. Proc. 11 Conf. Great Lakes Region 1968: 111-116.

Pfister, R.M., P.R. Dugan and J.I. Frea. 1969. Microparticulates: Isolation from water and identification of associated chlorinated pesticides. Science. 166: 878-879.

Pfister, R.M., J.I. Frea, P.R. Dugan, C.I. Randles, K. Zaebst, J. Duchene, T. McNair, and R. Kennedy. 1970. Chlorinated hydrocarbon, microparticulate effects on microorganisms isolated from Lake Erie. Proc. 13th Conf. Great Lakes Res. 1970: 82-92.

Postgate, J.R. and J.R. Hunter. 1962. The survival of starved bacteria. J. Gen. Microbiol. 29: 233-263.

Postma, H. 1954. Hydrography of the Dutch Wadden Seas. Arch. Neerl. Zool. 10: 1-106. In Sears (ed.) Progress in Oceanog. The MacMillan Co. p. 220.

Rasmussen, L. and T.A. Kludt (1970) Particulate material as a prerequisite for rapid cell multiplication in Tetrahymena cultures. Expl. Cell. Res. 59: 457-463.

Renn, C.E. 1963. The Bacteriology of Interfaces. In H. Heukelekian and N.C. Dondero (eds.). Principles and applications in aquatic microbiology. John Wiley and Sons, Inc., N.Y.

Seki, H. 1970. Microbial biomass on particulate organic matter in sea-water of the euphotic zone. Appl. Microbiol. 19: 960-962.

Shehata, T.E. and A.G. Marr. (1971) Effect of nutrient concentration on the growth of E. coli. J. Bacteriol. 107: 210-216.

Siegel, A. 1967. A new approach to the concentration of trace organics in seawater. In T.A. Olson and F.J. Burgess (eds.) Pollution and marine ecology. Intersci. Publ., N.Y.

Sieskind, O. and R. Wey. 1959. Sur l'adsorption d'acides amines par la montmorillonite. Compt. Rend. 248: 1652-1655.

Standard Methods for the examination of water and wastewater. 1960. American Public Health Assoc., Inc. N.Y., N.Y. pp. 245-251.

Vallentyne, J.R. 1954. Biochemical Limnology. Science. 119: 605-606.

Vellentyne, J.R. 1956. The molecular nature of organic matter in lakes and oceans, with lesser reference to sewage and terrestrial soils. Can. Fish. Res. B.J. 14: 33-82.

Van Marion, C. 1966. Oxidation of sodium lauryl sulfate, alkyl benzene sulfonate and chemically related compounds by Alcaligenes faecalis. Ph.D. Thesis. The Ohio State University.

Waksman, S.A. and C.L. Carey. 1935. Decomposition of organic matter in seawater by bacteria. J. Bacteriol. 29: 531.

Weber, J.B. and H.D.Coble. 1968. Microbial decomposition of diquat adsorbed on montmorillonite and kaolinite clays. J. Agri. Food Chem. 16: 475-478.

Williams: P.M. and L.I. Gordon. 1970. Carbon-13-carbon 12 ratios in dissolved and particulate organic matter in the sea. Deep Sea Res. 17: 19-27.

Wuhrmann, K. 1963. River bacteriology and the role of bacteria in self-purification of rivers. In Principles and applications in aquatic microbiology, Proc. Rudolphs Res. Conf. John Wiley and Sons, N.Y. pp. 167-192.

Zobell, C.E. 1943. Effect of solid surfaces upon bacterial activity. J. Bacteriol. 46: 39-56.

Part 7

Response of a pure culture of *Anacystis nidulans* and a unialgal culture of *Microcystis aeruginosa* to Aldrin and Dieldrin.

Part 7

LIST OF ILLUSTRATIONS

LIST OF TABLES

INTRODUCTION

There has been a growing concern with the role pesticides play in the ecosystem. Because of their recalcitrant nature, chlorinated hydrocarbon pesticides have become important subjects of study in the biosciences. Most work has been done on soil microorganisms and vertebrates such as birds and mammals. In contrast, there are few studies on the actions of pesticides on algae, and most of these have dealt with the effects of pesticides on marine phytoplankton. Attention was focused on phytoplankton organisms because of their importance as primary producers and also because they are the main source of the world's supply of oxygen.

Interest in pesticide-blue-green algal interactions arose as a result of research conducted by this laboratory in Lake Erie. Each year several "blooms" of blue-green algae appear in the region of the Bass Islands. Since these organisms contribute significantly to the flora of the lake, a question was posed concerning the effect of pesticides (shown by others to occur in the lake) on blue-green algae. This question was approached from two main areas: physiology and ultrastructure. Experiments were designed to correlate growth capabilities with photosynthetic rates. It was hoped that perhaps the mechanisms of pesticide action might be discovered. Moreover, any physiological changes might be reflected in ultrastructural alterations.

Trivial names of pesticides will be used throughout the text. Chemical formulae may be obtained from Menzie (1969).

REVIEW OF THE LITERATURE

Interactions Between Pesticides and Microorganisms

Pesticides are important ecologically because many are unnatural additions to the environment. Their fate in the ecosystem depends on the chemical and physical nature of the residues, environmental factors, and biological degradation (Van Middelem, 1966). The persistence of this type of compound can vary considerably. For example, the half-life (T 1/2) of the highly toxic organophosphates malathion and parathion is 1 week, while chlorinated hydrocarbons may remain for years. The T 1/2 for 1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl)ethane (DDT) is 4 yr; chlordane is 5 yr (Kearney, Nash, and Isenesee, 1969).

There are two areas of concern: one is the direct impact on biological systems and the other is an indirect impact due to bioconcentration. In the case of avian physiology the concentration of pesticides found in the soil has no effect. Their concentration through the food chain to higher trophic levels has affected avian reproduction severely (Wurster, 1969). Thus the balance of the environment may be affected at the lower trophic levels or after concentration at the higher levels.

Bioconcentration

A prerequisite to metabolism or increasing concentrations up the food chain is the ability of microbial bioconcentration. Chacko and Lockwood (1967) studied the accumulation of DDT and dieldrin by various microorganisms. Generally fungi and Streptomyces took up more pesticide, 70% or greater, than bacteria.

Floc forming bacteria, isolated from Lake Erie, have been shown to concentrate pesticides significantly (Leshniowski et al., 1970). These authors proposed that the accumulating flocs settle to the lake bottom removing pesticide containing material from the water column.

Bioconcentration of pesticides has been demonstrated for both fresh water algae (Gregory, Reed and Priester, 1969; Vance and Drummond. 1969) and marine phytoplankton (Keil and Priester, 1969; Cox, 1970 a, b).

Cox (1970a) reported that concentrations of DDT and related residues in marine phytoplankton increased 3 fold over the period 1955 to 1969. He suggested that this increase may be affecting the coastal pelagic food chain.

Södergren (1965) reported the uptake in Chlorella to be the result of passive absorption. His results indicated that maximum levels were reached within 15 sec after addition of the pesticide. Contrarily, maximum absorption of dieldrin to the same organism was achieved after 6 to 24 hr exposure (Wheeler, 1970). This difference can be accounted for by the solubilities of the two in water, the rate of absorption being inversely proportional to the solubility (Hill and McCarty, 1967), or the affinity of the absorbant for a specific chemical on the organism.

Metabolism

Some pesticides are relatively resistant to biodegradation or biodetoxification and have been termed "recalcitrant" (Alexander, 1967). Because of their structure these recalcitrant molecules may not be metabolized in the sense that they may serve as energy sources. However, they may be "co-metabolized". That is, pesticides may be partially degraded or detoxified by microorganisms without serving as energy sources.

Hill and McCarty (1967) examined the efficacy of anaerobic conditions in degrading chlorinated hydrocarbons. Of the pesticides tested (DDT, aldrin, heptaclor, heptachlor epoxide, dieldrin, 1,1-dichloro-2,2-bis(chlorophenyl)ethane(DDD), lindane and endrin) all but heptachlor epoxide and dieldrin were degraded. The latter also persisted under aerobic conditions. Ko and Lockwood (1968) suggested that anaerobic conditions enhanced by the addition of alfalfa were responsible for the increased rates of conversion of DDT to DDD by soil microorganisms. Patil, Matsumura and Boush (1970) showed that 22 soil microorganisms were capable of degrading DDT and endrin anaerobically while 13 isolates degraded aldrin aerobically.

A marked decrease in lindane concentration was effected by 2 species of green algae (Sweeney, 1965). The metabolite was shown to result from dechlorination to the non toxic

penta-chlorocyclohexene. Time course studies with Anacystis nidulans indicated that metabolism of lindane and DDT followed concommitantly with growth (Moor and Dorward, 1968).

Thus metabolism of pesticides appears to be dependent on the microbial isolate, the test environment and the type of pesticide.

Effect of Growth

In his review, Bollen (1961) was unable to find any consistent trends on the effect of pesticides on soil microorganisms. He noted that the application of a number of chlorinated hydrocarbons at maximal rates used on fields had no demonstrable effects on bacteria. Similar observations were obtained by Pfister et al. (1970). In surveying 151 heterotrophic isolates from Lake Erie, 55 cultures were stimulated by aldrin, 54 by endrin, and 45 by dieldrin. A negative effect was exerted on 46 cultures by aldrin, 43 by endrin and 43 by dieldrin.

Toxicity levels of pesticides to algae vary considerably with the pesticide and the test organism (Ukeles, 1962; Vance and Drummond, 1962). In many cases the toxicity levels for algae may range many fold above the solubilities of the pesticides in water.

A number of workers (Pierce, 1958; Pierce, 1960; Tatum and Blackburn, 1962; Sweeney, 1968) have suggested that while algae may be adversely affected initially they are capable of adapting to the pesticide.

In contrast Sodergren (1968) showed that Chlorella sp. grown in continuous culture was severely inhibited by 0.6 ng/ml DDT. Similarly Menzel, Anderson and Randtke (1970) reported that daily addition of 0.1 μg/ml endrin inhibited 3 of 4 species of phytoplankton investigated while DDT at the same concentration inhibited 2 species. Also, a single addition of DDT, toxaphene and dieldrin to Scenedesmus cultures caused a decrease in cell numbers and biomass; carbaryl and diazinon either had no effect or enhanced cell division (Stadnyk, Campbell and Johnson, 1971).

Effect of Physiological Parameters

In addition to noting the effect of pesticides on growth, short term experiments utilizing carbon fixation and oxygen production have been reported.

An early study (Anonymous, 1963) indicated that carbon fixation in marine phytoplankton was reduced greater than 75 per cent by a number of pesticides. More recently, pesticides have been shown to reduce carbon fixation (Wurster, 1968; Menzel, Anderson and Randtke, 1970) and oxygen evolution (Derby and Ruber, 1971) of marine phytoplankton. Some comparisons can be made of the latter 3 studies, since the authors employed a similar organism (Skeletonema costatum), the same concentrations of DDT, and similar conditions. Wurster (1968) reported 0.1μg/ml DDT reduced carbon fixation about 75%. Menzel, Anderson and Randtke (1970) reported a 35% reduction in ^{14}C uptake while oxygen evolution was inhibited to 32% of the control (Derby and Ruber, 1971). While there is some discrepancy as to the

extent of inhibition, these studies confirm the fact that basic physiological functions can be affected. However, as shown by these workers, the effect is pesticide-, concentration-, and species-dependent.

In situ studies were performed on the effect of DDT and dieldrin on carbon fixation in freshwater phytoplankton (Glooschenko, 1971). On 4 stations located in Lake Erie even the lowest concentrations (1 ng/ml) used inhibited ^{14}C uptake. At higher concentrations increased inhibition took place. These short term experiments (approximately 20 hr) provide only an indication of the initial effects. Stadnyk, Campbell and Johnson (1971) presented evidence of recovery of carbon fixation after extended incubation. They caution that "codistillation" (Acree, 1963) of the pesticide with evaporation of the medium may play an important part in this recovery process.

In summary, some general observations can be drawn about pesticide-microorganism interactions: i) Pesticides may be absorbed to microorganisms inversely proportional to their solubilities. The absorption process appears to be passive. ii) Pesticides may serve as carbon sources or may be degraded through the mechanism of cometabolism. iii) Physiological processes, such as carbon fixation and oxygen production, may be affected by pesticides. Such an effect may be reflected in altered growth patterns. iv) The effect on organisms is pesticide-, concentration- and species-dependent. v) Adaptation to toxicants, at least in algae, may provide for the restoration of environmental balance.

Ultrastructure of Blue-green Algae

Over the past 10 to 15 yr extensive studies have been made of the ultrastructure of blue-green algae. These algae may be characterized as primarily photolithotrophs although slight growth can be obtained heterotrophically in the dark. They possess only chlorophyll a in their oxygen-evolving photosynthetic apparatus. Moreover, they are more similar to procaryotes than eucaryotic organisms in their structure, biochemistry, and ecology.

Cell Envelope

Sheath. --The outermost structure of the cell is a mucilaginous sheath. Its morphology varies from species to species, and may in some cases be absent. The fine structure of the sheath is dependent upon the types of fixatives and dehydration procedures employed. Generally, the sheath is considered to be extracellular since it is outside the cell wall (Lang, 1968). The sheath represents an excellent substrate for bacteria often complicating the production of axenic cultures (Lang, 1968; Tuffery, 1969).

In their study of 13 axenic cultures of blue-green algae, Ris and Singh (1961) state that the ultrastructure of the sheath "appears as a mass of oriented interwoven fibrils." These fibrils are in contact with the cell wall and are zonal becoming more diffuse farther from the cell. The sheath of Symploca muscorum is composed of two layers. The inner dense zone measures from 100-130 nm, while the outer less dense layer ranges from 100-200 nm (Pankratz and Bowen, 1963). Chlorogloea fritschii demonstrated a sheath similar to that of S. muscorum (Peat and Whitton, 1967). A micro-fibrillar network comprised the sheath of a Nostoc species. These fibrils appeared compressed into stria near the cell

becoming diffuse distally (Tuffery, 1969).

The sheath of an Anabaena species was described as an undifferentiated structure with fibrils radiating out from the cell wall (Leak, 1967). Lamont (1969) described the sheath of two motile oscillatoracean blue-green algae. The screw-type motion of these organisms, the author suggested, is responsible for the orientation of the microfibrils roughly parallel to the stream lines on the surface of each organism.

Some species, such as Anacystis montana f. minor, possess a very thin sheath (Echlin, 1969 a). There is some question as to whether Gleocapsa alpicola and Anacystis nidulans possess a sheath although Allen (1968 b) states that the electron dense layer is a sheath.

The chemical composition is polysaccharide in nature (Lang, 1968; Tufferty, 1969).

Cell wall and plasmalemma. - To our knowledge all blue-green algae, both freshwater (Ris and Singh, 1961; Pankratz and Bowen, 1963; Echlin, 1964a; Peat and Whitton, 1967; Allen, 1968b; Gantt and Conti, 1969) and marine (Van Baalen and Brown, 1969), demonstrate the same cell wall profile. Originally the wall was described as having an outer tripartite layer and an inner dense layer bordered by the plasma membrane (Ris and Singh, 1961; Pankratz and Bowen, 1963) reminiscent of the gram negative cell wall of bacteria (Edwards et al., 1968). The undulated outer layer (17 nm) is separated from the inner dense layer by a 3 nm electron transparent space. The plasmalemma, a typical unit membrane (7 nm), is separated from the inner dense layer by an irregular electron transparent layer of a few nm. The membrane also exhibits undulations but not as regular as the outer layer.

Lang (1968) applauds Jost (1965) for his numerical labelling of the various layers. Since more than the outer, inner and plasmalemma layers have been identified she feels this approach to be the more feasible. According to the terminology of Jost (1965) the layers are numbered in sequence distally from the plasmalemma. Thus the L I layer is the electron transparent area bordering outside the membrane; L II is the inner dense layer; the electron transparent layer separating the inner dense portion from the outer tripartite structure is the L III; the outer tripartite structure is L IV; beyond L IV is the sheath. Allen (1968 b) employed this terminology but suggested that the L I layer may be an artifact of preparation. This is evidenced by the fact that the width is irregular from cell to cell and within the same cell; the membrane in some cases is appressed to the L II layer.

Chemical studies on the structure of the cell wall indicate a peptidoglycan of composition similar to that of bacteria (Drews and Meyer, 1964), and localized in the L I layer. Should Allen (1968 b) be correct in her assertion that L I is an artifact, then perhaps L II is the peptidoglycan.

Thylakoids

It is believed that the thylakoids contain the photosynthetic apparatus. the lamellae of these structures are composed of two membranes which enclose a 35 nm intralamellar space (Lang, 1968). The membranes are typically 7 nm and within the space bounded by

these membranes are intralamellar vesicles of low density; outside can be found α granules (to be described) and ribosomes (Ris and Singh, 1961; Pankratz and Bowen, 1963; Echlin, 1964 a). In permanganate fixation, the lamellae appear as either five or seven layers of alternating electron transparent and electron dense bands (Echlin, 1964a).

Lamallae probably are synthesized de novo although in some cases they appear to result as an elaboration of the plasmalemma (Pankratz and Bowen, 1963; Edwards et al., 1968). Others (Van Baalen and Brown, 1969) have suggested that the cylindrical bodies, first reported by Pankratz and Bowen (1963), may be the site of thylakoidal synthesis in a marine blue-green alga.

The thylakoids may be arranged peripherally in concentric shells (Ris and Singh, 1961; Echlin 1964a; Edwards et al., 1968) longitudinally (Gantt and Conti, 1969) or randomly (Pankratz and Bowen, 1963).

Environmental conditions may affect the thylakoidal arrangement. In young cells of Chlorogloea fritchii thylakoids form concentric shells but cultures in the dark with sucrose for three years demonstrated lamellae scattered throughout the cell (Peat and Whitton, 1967). Allen (1968 a) showed that pigment concentration and lamellar content varied inversely with light intensity.

Phycobilisomes. --These inclusions, located adjacent to the thylakoids, were described in a red alga and shown to contain the biliproteins, phycoerytherin and phycocyanin (Gantt and Conti, 1967). Since then this term has been used to describe similar structures in blue-green algae (Edwards et al., 1968; Gantt and Conti, 1969).

Nucleoplasm

Blue-green algae possess a nuclear region not unlike that of bacteria. The nucleoplasm is not bounded from the rest of the cell by a nuclear membrane. The nucleoplasm is visualized as a polymorphous structure of low density containing 2.5 nm fibrous material in an electron transparent matrix (Ris and Singh, 1961; Pankratz and Bowen, 1968; Echlin, 1964a). Most of this material is located in the central portion of the cell although it may be formed peripherally. A positive feulgen reaction in the central region strongly suggests the presence of deoxyribonucleic acid (DNA) (Pankratz and Bowen, 1963; Echlin, 1964a).

Cellular Inclusions

Cyanophycin granules. --These granules occur in a variety of blue-green algae. They appear as a spherical mass of membranes wound in concentric circles (Echlin, 1964 a) or as closely arranged undulating membranes (Ris and Singh, 1961; Pankratz and Bowen, 1963). Known also as "structured granules" (Ris and Singh, 1961; Pankratz and Bowen, 1963) or "lamellasomes"(Echlin, 1964 a), they have been shown to be associated with the plasmalemma of the septum and the thylakoids. Lang and Fisher (1969) have demonstrated the variation in structure of these granules to be due to the fixation procedure.

Because of their similarity to mesosomes Drews and Niklowitz (1957) considered them

to be "mitochondrial equivalents." However, Bisalputra, Brown and Weier (1969) demonstrated that these structures reduce neither tellurite or tetranitro-blue tetrazolium. Their chemical structure and precise biochemical function await further investigation (Lang, 1968).

Polyphosphate granules. --These granules apparently are reserve storage areas of phosphate 30-400 nm in diameter. They are variously known as volutin and metachromatic granules (Lang, 1968). Their chemical nature is of several types; polyphosphates (8 residues), oligophosphates (2-7 residues) and circular metaphosphates (Niemeyer and Richter, 1969).

Jensen (1969) described the developmental sequence of these inclusions in Plectonema boryanum. Initially an electron translucent area 150-200 nm diameter appeared in the cytoplasm. Then two events occurred nearly simultaneously. Electron dense material was deposited in the electron transparent area to produce a porous body in thin sections. At the same time, in the adjacent cytoplasm, deposition of an electron dense material took place. Following this electron dense polyphosphate was deposited centrifugally into the porous areas of the granule. In the final stage the entire granule became electron dense.

Polyhedral bodies. --In her review, Lang (1968) suggested that these bodies had been mistaken by some authors for polyphosphate granules and did not include them in her discussion. Subsequently (Lang and Fisher, 1969) her micrographs have included the labelling of such structures.

These large medium dense bodies, with more or less polygonal profiles, have been described in a number of species (Pankratz and Bowen, 1963; Peat and Whitton, 1967; Edwards et al., 1968; Van Baalen and Brown, 1969). They are located primarily in the region of the nucleoplasm and range up to 500 nm in diameter. Gantt and Conti (1969) described a similar body with a helical pitch spanning the entire length of a dividing cell of Anacystis nidulans. Furthermore, negatively stained bodies from disrupted cells showed them to be composed of small subunits in a crystalline array. Upon closer examination, these subunits appear to be hexagonal.

Polyglucoside granules. --These structures were first described as αgranules 30 nm in diameter. They are of medium-electron density and are found adjacent to the thylakoidal membranes (Pankratz and Bowen, 1963; Echlin, 1964). Rod shaped aggregations are characteristic of Oscillatoria chalybia. Diastase digestion of these granules suggests their polyglucoside content (Giesy, 1964). In Anacystis nidulans these granules were selectively digested by αamylase (Lang, 1968).

Lipid globules. --These osmophilic inclusions range in size from 30 to 90 nm and are found primarily among the lamellae near the cross walls of dividing cells (Pankratz and Bowen, 1963). The term globules was coined by these authors and has been used by others (Bisalputra, Brown and Weier, 1969).

Ribosomes. --Ribosomes are electron dense structures 10 to 15 nm in diameter. They occur primarily on the periphery of the nucleoplasm although some appear among the lamellae of the photosynthetic apparatus (Ris and Singh, 1961; Pankratz and Bowen, 1963; Echlin, 1964 a). Ris and Singh (1961) based their interpretation i) on the similarity of

ribosomes from bacteria to those from green algae and ii) histochemical results of staining with pyronine before and after ribonuclease treatment. Ordered rows of up to 15 granules have been observed with freeze-etching and also probably represent ribosomes (Jost, 1965).

Gas Vacuoles. --Under light microscopy, gas vacuoles can be seen as reddish structures which disappear on application of pressure (Smith and Peat, 1967 a). Gas vacuoles occur primarily in older cells, although Waal and Branton (1969) induced their formation in distilled water. They may occupy as high as 39%of the volume in some cells (Smith and Peat, 1967 a, b).

Electron microscopic examination of these structures reveals that they resemble gas cylinders approximately 70 nm in diameter ranging from 100 to 1300 nm in length depending on their stage of development (Smith and Peat, 1967 a, b; Waal and Branton, 1969; Jost and Jones, 1970).

The boundaries of the gas cylinders are "membranes" with a thickness of 2 to 3 nm (Smith and Peat, 1967 a; Jost and Jones, 1970). Chemical analysis of isolated membranes indicated that they are proteinaceous only (Smith, Peat and Bailey, 1969; Jones and Jost, 1970). In thin sections the gas vacuole membrane shows only a single track (Smith and Peat, 1967 a) appearing to represent half a unit membrane.

Furthermore these "half membranes," when frozen-etched or negatively stained, are made up of ribs with a spacing of 4.0 to 5.0 nm (Smith, Peat and Bailey, 1969; Jost and Jones, 1970). Some observations suggest that the ribs form a helix. Making up the ribs of the membrane are globules separated by approximately 3.5 nm (Smith, Peat and Bailey, 1969; Jost and Jones, 1970).

In frozen-etched cells the spacing between the ribs often shows a discontinuity which Waal and Branton (1969) have interpreted as a growing point. Jost and Jones (1970) prefer to interpret them as artifacts due to plastic deformation during the etching process.

In addition to blue-green algae, the gas cylinder is a morphological entity in some purple as well as green and non photosynthetic bacteria (Cohen-Bazire, Kunisawa and Pfennig, 1969).

Other inclusions. --A number of unusual, and seldom if ever before described, structures have been reported by Jensen and Bowen (1970).

Growth

Allen and Stanier (1968) reported on the growth of 6 blue-green algae using time-lapse photomicrography. The development of rod shaped organisms in slide culture was similar. Using the rod shaped Anacystis nidulans as an example, the authors showed that cell division takes place perpendicular to the longitudinal axis of the cell. Gleocapsa alpicola, a spheroid laking a sheath, divided in planes at right angles to each other. Another ensheathed isolate, not identified by the authors, divided in three planes (at right angles to each other) to yield a three dimensional 8-celled colony.

The participants in crosswall formation have been known for some time to be the plasma membrane, and L I and L II layers (Ris and Singh, 1961; Pankratz and Bowen, 1963; Echlin, 1964a: Allen, 1968 b). Allen (1968 b) described in detail the ultrastructure of cell division of Anacystis nidulans and Gleocapsa alpicola. Unlike Anacystis montana f. minor (Echlin, 1964 a), A. nidulans does not constrict prior to cell division. Septum formation in A. nidulans proceeds by an invagination of the plasma membrane with a concurrent centripedal proliferation of the L II layer (recall that the L I layer may be an artifact). Once the invaginating membranes anastamose they separate forming the plasma membrane of each new daughter cell; the division aperture is then sealed with the fusion of the L II layer. The L III and L IV regions, which are not direct participants in cross wall formation, grow inward separating the L II layer equally for each new daughter cell. Synthesis of the sheath follows in sequence.

Generally constriction of the cytoplasmic contents, the lamellae and nucleoplasm, occurs prior to their separation by the septum. However, in A. nidulans there is a differential asymetric invagination of the thylakoids preceding the onset of cell division

G. alpicola divides in essentially the same manner, its lamellae being constricted. On division in one plane, the initiation of septum formation at right angles can be discerned.

MATERIALS AND METHODS

Culturing of cells

Organisms. --Two blue-green algae were employed in this study. Anacystis nidulans was obtained as an axenic culture from the Indiana University Culture Collection (IU 625). This strain is a high temperature mutant (optimum temperature of approximately 41 C) and was originally isolated by Kratz and Allen (Starr, 1964).

Using a modification of the technique of Allen and Stanier (1968), Johnson (1970) isolated the other blue-green alga from Lake Erie. This isolate has been tentatively identified as Microcystis aeruginosa. The culture appeared to be unialgal although contaminated with bacteria.

Maintenance of Cultures. --A. nidulans was maintained on cotton plugged "Cyanophycean Agar" slants described by Starr (1964). The components were as follows: KNO_3, 5.0 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05g; Fe^{+3} ammonium citrate, 10 drops of a 1% solution; agar (Difco), 15 g per 1000 ml deionized double distilled water. The salts and agar were autoclaved (15 psi, 121C, 15 min) separately.

Cells were grown at ambient temperature (approximately 28 C) under room lights producing 100 foot-candles (ft-cd).

M. aeruginosa did not lend itself well to culturing on agar slants. Therefore, the culture was maintained in the growth medium described below, under the same environmental conditions as A. nidulans.

Growth medium. --The medium employed for growth studies was that cited by Allen and Stanier (1968). It contained: K_2HPO_4, 0.039 g; $MgSO_4 \cdot 7H_2O$, 0.075 g; Na_2CO_3, 0.020 g; $CaCl_2 \cdot 2H_2O$, 0.027 g; $Na_2SiO_3 \cdot 9H_2O$, 0.058 g; ethylenediaminetetracetic acid (EDTA), 0.001 g; citric acid, 0.006 g; $NaNO_3$, 1.5 g; microelement mixture A5, 1 ml per 1000 ml deionized double distilled water. Microelement mixture A5 contained H_3BO_4, 2.86 g; $MnCl_2 \cdot 4H_2O$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $NaMoO_4 \cdot 2H_2O$, 0.391 g; $CuSO_4 \cdot 5H_2O$, 0.079 g; $Co(NO_3)_2 \cdot 6H_2O$, 0.0494 g per 1000 ml deionized double distilled water.

The medium was distributed as 50-ml quantities into 250-ml flasks stoppered with cotton plugs and autoclaved as above.

Growth conditions. --One ml from a 7-day preculture of A. nidulans was used to inoculate the test flasks. The cultures were incubated at 37C on a Psychrotherm (New Brunswick) rotary shaker at 120 rpm. Illumination was provided by an overhead bank of cool white fluorescent lamps at an incident intensity of approximately 114 ft-cd.

A 14-day preculture of M. aeruginosa provided 1-ml inocula for the 250-ml test flasks. The cultures were incubated at ambient temperature (28 ± 2 C) on a rotary shaker at 120 rpm. Overhead illumination (87 ft-cd incident light intensity) was provided by cool white fluorescent lamps.

Measurement of Physical Parameters

Cell numbers. -- Cell numbers were determined using a Petroff-Hauser counting chamber.

pH. --The pH of the culture was measured with a Corning model 12 pH meter (Corning, New York).

Chlorophyll a. --Chlorophyll a (chl a) was quantitated by the methanolic extraction procedure of Holden (1965). A 10-ml aliquot of cells was centrifuged and then resuspended in methanol. After recentrifugation, the supernatant fluid containing chl a was decanted. The absorbancy of the pigment was measured on either a Gilford Spectrophotometer 2400 (Gilford, Oberlin, Ohio) or a Shimadsu MPS spectrophotometer (American Instrument, Silver Spring, Md.). The pigment was quantitated according to the formula: μg chl a/ml = $16.5A_{665nm} - 8.3_{650nm}$.

Oxygen evolution. --To estimate rates of photosynthesis, a 1.5-ml aliquot of culture was dispensed into a water-jacketed cuvette (Gilson Medical Electronics, Middleton, Wis.) fitted with a Clark oxygen electrode (Yellow Springs Instrument, Yellow Springs, Ohio) and a magnetic stirring bar. Oxygen evolution at 27 C was measured upon illumination with an incandescent lamp at 5000 ft-cd incident light intensity.

Pesticides

Addition to the culture. -- After inoculation of the medium, the pesticides aldrin (1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 8, 8a-hexahydro-1, 4-endo-exo-5, 8 dimethanonaphtalene) or dieldrin (1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7,

8a-octahydro-1, 4-endo-exo-5, 8-dimethanonaphthalene) were added in 0.1 ml distilled acetone to a final concentration of 6.5 μg/ml. Acetone (0.1 ml) which contained no pesticide was added to the control flasks. The same procedure was employed for daily additions.

Extraction from the culture. --Three fractions were examined for pesticide content. These were the supernatant, hexane (to remove adsorbed pesticide) and cell fractions.

Ten ml of culture was withdrawn and centrifuged to remove the cells. The supernatant fraction was then decanted and saved for later analysis.

The pellet was resuspended in 5 ml hexane and shaken with a vortex mixer for 5 sec. After centrifugation the hexane was decanted and stored. This procedure was included to remove pesticides merely adsorbed to the surfaces of the cells. Since no alteration in the cell structure could be observed under phase contrast microscopy, it was assumed that the cells' integrity was maintained.

The pellet was again suspended in 5 ml water. The suspension was subjected to sonification for 15 min at a setting of 80 on a Biosonic III(Bronwill Scientific, Rochester, N.Y.). Temperature was maintained at 25 C with a water jacket fitted to the test tube. Microscopic observation of the suspension after sonification revealed only cell debris (99% disruption).

Quantitation. --The supernatant and cell fractions were subjected to further extraction into hexane; the hexane wash was not included.

Extraction was accomplished in a teflon stopcock separatory funnel. Samples were poured into the funnel. An equal volume of hexane was poured into the sample container, swirled and added to the funnel. After shaking for 1 min, the funnel was allowed to sit for 10 min. The water phase was drawn off into the sample container and the hexane was drawn off into a beaker. This procedure was repeated 3 x for each sample. Following the final extraction, the funnel was rinsed with hexane which was added to the beaker.

The total of the 3 extractions and the rinse was evaporated and resuspended in hexane to a known volume (2 ml). With this procedure the samples were ready for quantitation.

A check of the loss of pesticide due to the evaporation step revealed little or no change. For aldrin the percent recovery was 99% for the evaporation of 10 ml to 1 ml; for dieldrin, 90%. Each fraction was treated exactly the same so that any errors due to evaporation remained constant.

Analysis of the hexane extracts was made using an Aerograph 200 gas chromatograph (Wilkens Instrument, Walnut Creek, Calif.) equipped with an electron capture detector (250 mc titanium tritritide). The 5-ft glass column (0.147 in I.D.) was packed with Chromosorb W 60/80 mesh, coated with 5% Dow silicone SE-30 (Applied Science, State College, Pa.). Column temperature was maintained at 190 C; the detector at 200 C; and the injection port at 230C. The carrier gas was high purity N_2.

Electron Microscopy

Cells were prepared for electron microscopy by employing a double fixation technique wherein glutaraldehyde serves as the prefixative and osmium, the postfixative. In order to highlight cellular structure, the cells were stained with uranyl acetate. Finally, after dehydration and embedding, sections of the material were poststained with lead citrate.

Fixation. --A 1-ml aliquot of 30% (v/v, in cacodylate buffer) glutaraldehyde was added to a 10-ml suspension of cells for 2 hr at ambient temperature. The buffer consisted of 100 ml 0.1 N Na-cacodylate and 8.3 ml of 0.1 N HCl yielding a final pH of 7.2 (Mercer and Birbeck, 1962). The suspension was then washed 3 x in cacodylate buffer and 2 x in acetate-veronal buffer.

Postfixation was carried out with the method of Kellenberger, Ryter and Sechaud (1958). The washed cell pellet was suspended in 1 ml 1% OsO_4 (w/v) in acetate-veronal buffer. Following exposure for 4 hr at room temperature, the cells were washed with 8-10 ml acetate-veronal buffer.

Acetate-veronal buffer (stock) was made as follows: Na-acetate, 19.4 g; Na-barbital, 29.4g; Na Cl, 34.0 g; distilled water to 1000 ml. The working solution was composed of 5 ml of the stock buffer, 7 ml 0.1 N HCl, 13 ml distilled water, and 0.25 ml 1 M $CaCl_2$. The final pH was 6.1.

In some cases the permanganate fixation of Luft (1956) was employed instead of the glutaraldehyde-osmium double fixation described above. Cells were suspended in 2% $KMnO_4$ (w/v in 42 ml veronal stock and 60 ml double distilled water) for 1 hr at 0-5 C and washed with 8-10 ml veronal-acetate buffer.

Following gluteraldehyde-osmium or permanganate fixation, cells were suspended in 2 drops 2% agar (w/v) in veronal-acetate buffer. After solidification of the agar, 1 mm cubes were cut. The cubes were washed with 5% uranyl acetate (w/v in veronal-acetate buffer) for at least 2 hr.

Dehydration. --Before embedding, the agar cubes were dehydrated in ethanol (EtOH) according to the following schedule: 50% EtOH, 25 min; 70% EtOH, 25 min; 2 changes 95% EtOH, 20 min each; 2 changes 100% EtOH 20 min each; 2 changes of 100% propylene oxide, 15 min each.

Embedding. --When cells were embedded in Epon, the cubes were placed in equal parts propylene oxide-complete resin monomer for at least 1 hr. The monomer mixture was that of Luft (1961): Mixture A was composed of 100 ml of dodecyl succinic anhydride (DDSA) and 62 ml Epon 812. Mixture B contained 89 ml methyl nadic anhydride (MNA) and 100 ml Epon 812. Fifty-five parts mixture A and 45 parts mixture B were added to 1.5% 2, 4, 6-tridimethylaminomethyl phenol (DMP-30). Extreme care was taken to ensure complete mixing of all components.

After propylene oxide-complete monomer exposure, the agar cubes were placed in

"beem" capsules filled with monomer. The agar blocks were allowed to settle to the bottom overnight at room temperature. Polymerization was carried out at 45 C for 12 hr and 60 C for 12 hr.

When embedding was carried out in Maraglas, the dehydrated cells were suspended in equal parts propylene oxide-Maraglas mixture for 30 min and then 1 hr in the Maraglas mixture alone. The Maraglas mixture was prepared according to Freeman and Spurlock (1962): Maraglas 655, 68 parts; Cardolite NC 513, 20 parts; dibutyl phthalate, 10 parts; benzyl dimethylamine (BDMA), 2 parts.

The cubes were placed in "beem" capsules containing fresh Maraglas mixture. The specimen cubes were allowed to settle (12 hr) at room temperature. Polymerization was completed after 48 hr at 60 C.

Sectioning. --Ultrathin sections (silver) were obtained with a diamond knife (Dupont, Wilmington, Del.) on an Ultratome III (LKB Broma, Sweden). The sections were collected on uncoated 300 mesh grids.

Post-staining. --Specimens were post-stained with lead citrate (Reynolds, 1963) for 30 min at 40 C in a dessicator containing NaOH pellets. The sections were rinsed with distilled water.

The stain was prepared by combining 1.33 g Pb $(NO_3)_2$ and 1.76 g Na-citrate with 30 ml distilled water in a 50 ml volumetric flask. The suspension was shaken vigorously for 1 min. Occasional shaking was continued for 30 min after which 8 ml of 1 N NaOH was added and the solution diluted to 50 ml with distilled water.

Electron microscope. --Sections were observed with a Phillips EM 300 at 80 kv.

RESULTS

Effect of Pesticides on Growth, Oxygen Production and Chlorophyll Content

Anacystis nidulans. --The results from a single addition of the pesticides, aldrin or dieldrin, are presented in Figs. 1-6. During the first 5 days incubation, both pesticides slightly inhibited the growth of A. nidulans (Fig. 1). A. nidulans, grown in the presence of aldrin, reestablished growth rates comparable to the control after 5 days. Dieldrin-grown cells, in contrast, increased in rate of cell division compared to the control. Final cell numbers approximated the control.

The pH of all three cultures remained at approximately 7 for 5 days, after which it rose to 10.3 (20 days). It should be noted that the medium was only slightly buffered (cf. MATERIALS AND METHODS). When all components of the medium were first mixed the pH was 10.

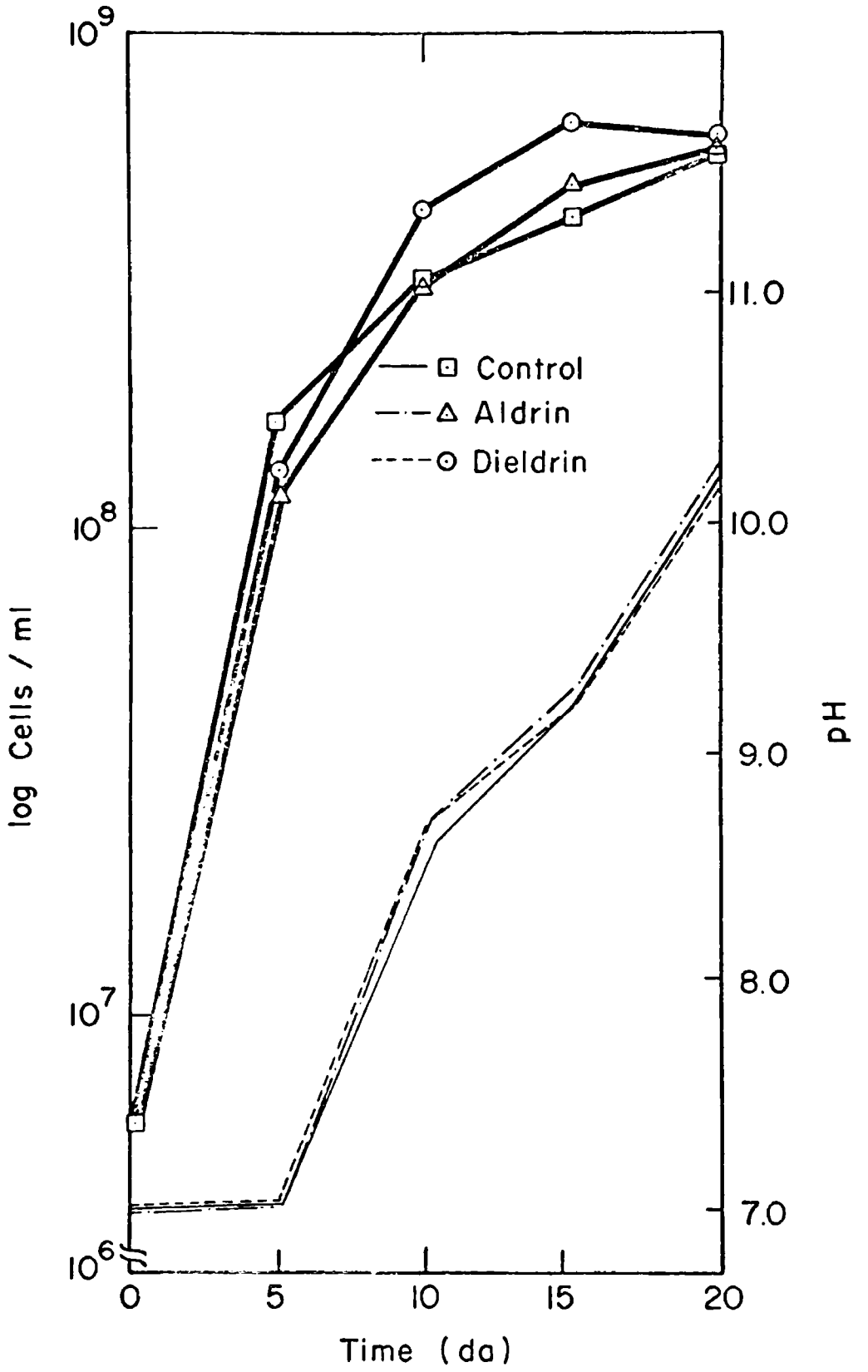

Fig. 1. -- Growth and pH changes of A. nidulans after a single addition of pesticide. Squares represent the control; triangles, aldrin-treated cells; circles, dieldrin-treated cells; solid lines, growth; dashed lines, pH.

Initially the oxygen evolving capacity of the cells treated with dieldrin was suppressed to 50% of the control (Fig. 2). Aldrin treated cells were only slightly inhibited in their photosynthetic rates. From 5 days, photosynthesis in all three treatments were relatively similar.

The efficacy of chl a to evolve oxygen is shown in Fig. 3. Dieldrin inhibited this function while aldrin was slightly stimulatory. After 5 days these rates were comparable to the control.

Throughout the 20-day growth period the chl a to cell ratio remained constant as demonstrated in Fig. 4.

Because of the nature of the above experiments, there was a lag of about 24 hr between the time of inoculation and the completion of the initial readings. For this reason experiments were done to determine immediate as well as 24 hr effects of the pesticides (Table 1). The immediate addition of aldrin demonstrated little effect on the rate of oxygen evolution, while the effect of dieldrin was slightly more pronounced. However, after 24 hr dieldrin suppressed photosynthetic rates considerably while rates for the aldrin treated cells did not differ from the control.

In another series of experiments (Figs. 5-8), pesticides (6.5 μg/ml) were added daily for 5 days. This was done to simulate the natural situation where there might be a continuous input of pesticide into the aquatic environment.

Figure 5 demonstrates the effect on growth of the daily addition of pesticides. Initially growth was slightly suppressed for the dieldrin-treated cells. Thereafter, growth continued at rates similar to the control. Growth again was adversely affected after days 4 and 5. Aldrin-treated cells maintained growth rates which did not differ from the control.

The ability of the cells to carry out photosynthesis was more severely inhibited by dieldrin than by aldrin (Fig. 6). Dieldrin-treated cells subsequently were not so affected as the aldrin-treated cells.

Although both pesticides suppressed oxygen production of the treated cells, only dieldrin greatly inhibited the ability of chl a to evolve oxygen (Fig. 7). Aldrin suppressed photosynthesis slightly but not to the extent of dieldrin.

Chl a of the cells was lower in the presence of pesticides during the initial stages of growth (Fig. 8). As development proceeded chl a content of dieldrin-treated cells returned to levels of the control more rapidly than cells treated with aldrin.

Microcystis aeruginosa. --The results of a single addition of pesticide to a culture of M. aeruginosa are presented in Figs. 9-12.

In growth studies over a 15-day period, aldrin affected growth more than dieldrin (Fig. 9). Aldrin inhibited growth initially (5 day), but the cell count rose to slightly above the control by the fifth day. Dieldrin did not appear to affect growth. Both aldrin- and dieldrin-treated cells maintained the pH at appreciably higher levels than the control

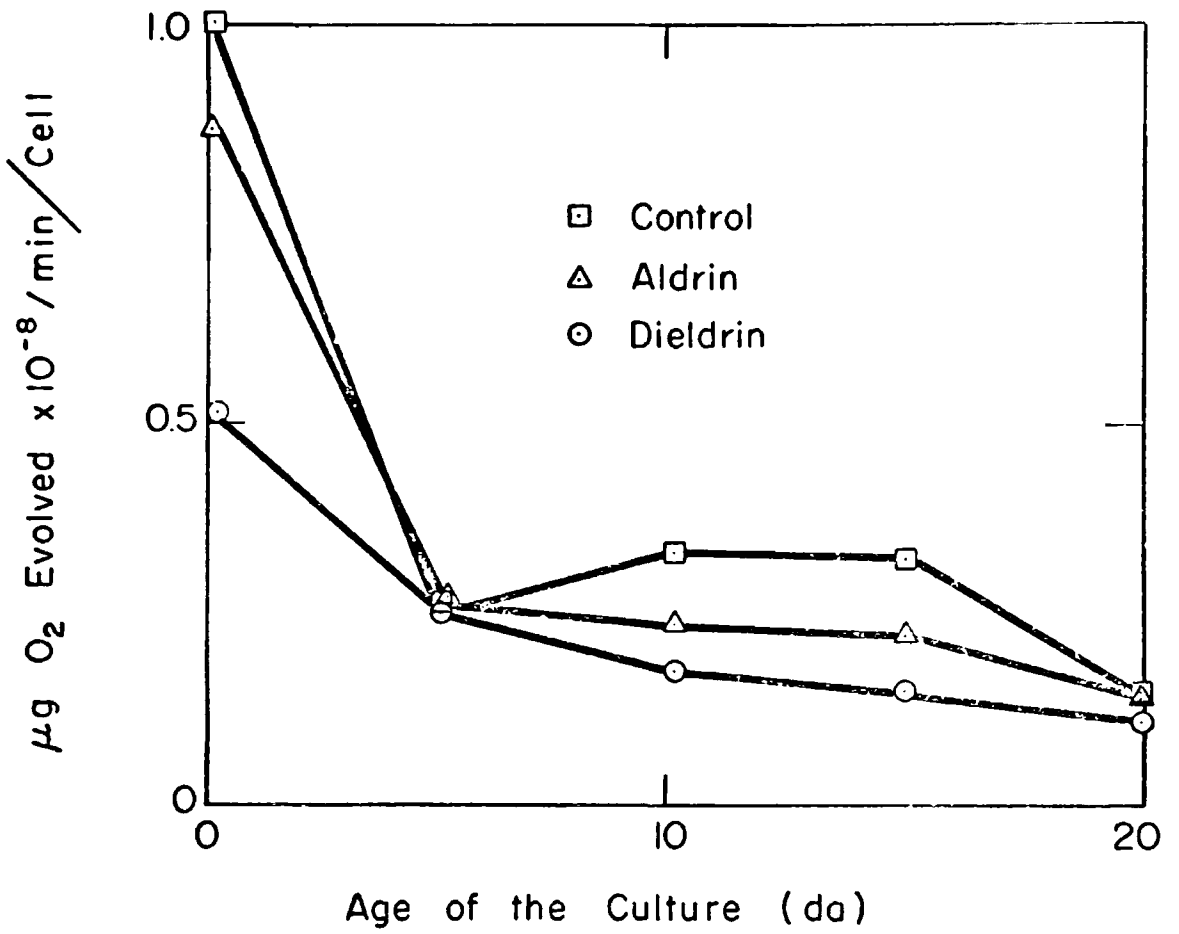

Fig. 2. -- Rates of photosynthesis per cell for A. nidulans after a single addition of pesticide. Squares represent the control; triangles, aldrin-treated cells; circles, dieldrin-treated cells.

TABLE 1.--Suppression of photosynthesis in A. nidulans initially and after 24 hr exposure to aldrin or dieldrin.[a]

Description	% of control	
	initial	after 24 hr exposure
Aldrin	94.5	100.0
Dieldrin	91.5	59.1

[a]Photosynthesis was based on the ug O_2 evolved/min/cell upon exposure to either 6.5 µg/ml aldrin or dieldrin.

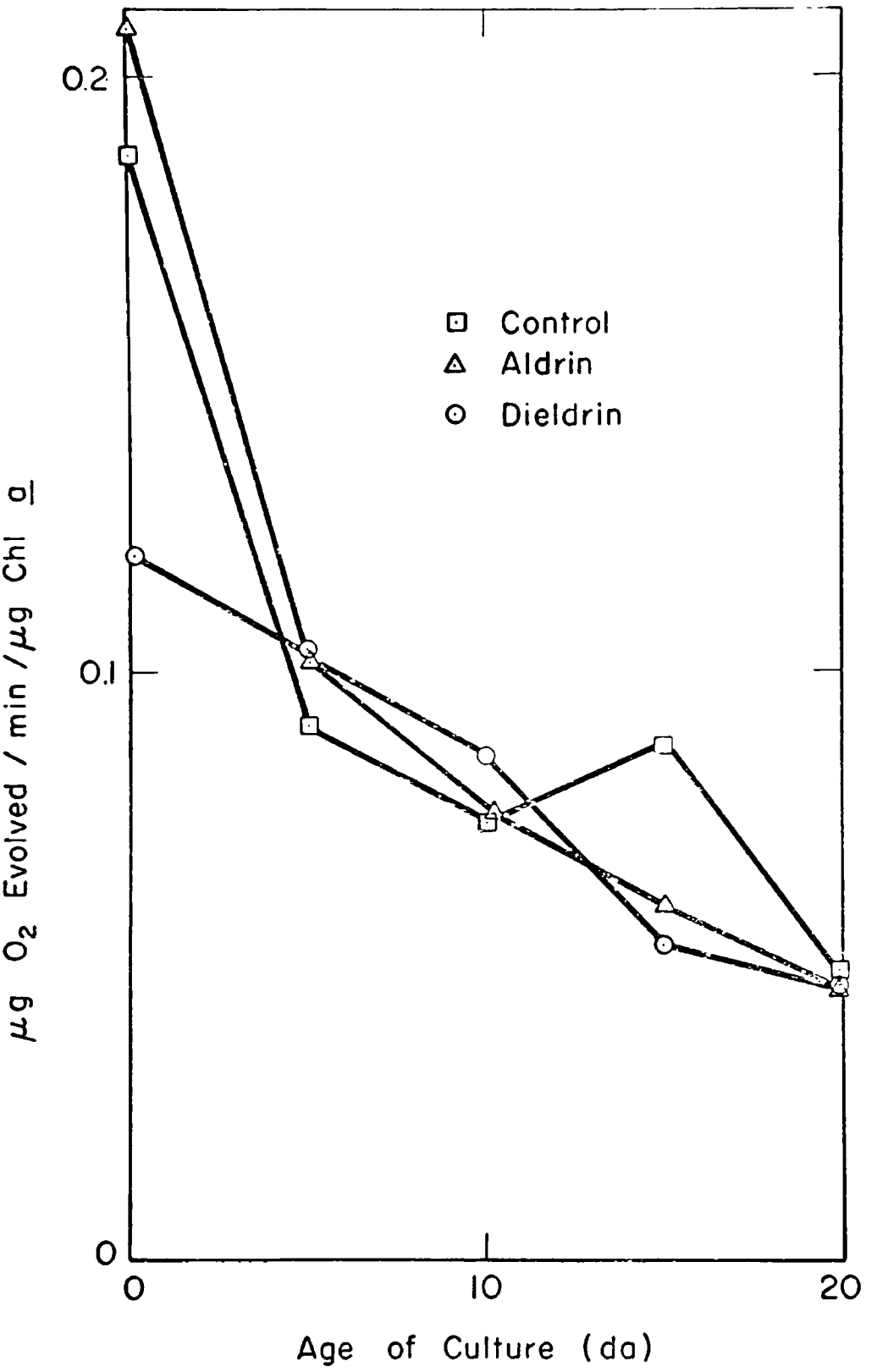

Fig. 3. -- Photosynthetic rates for A. nidulans based on chl a content. A single addition of pesticide was made initially. Squares represent the control; triangles, aldrin-treated cells; circles, dieldrin.

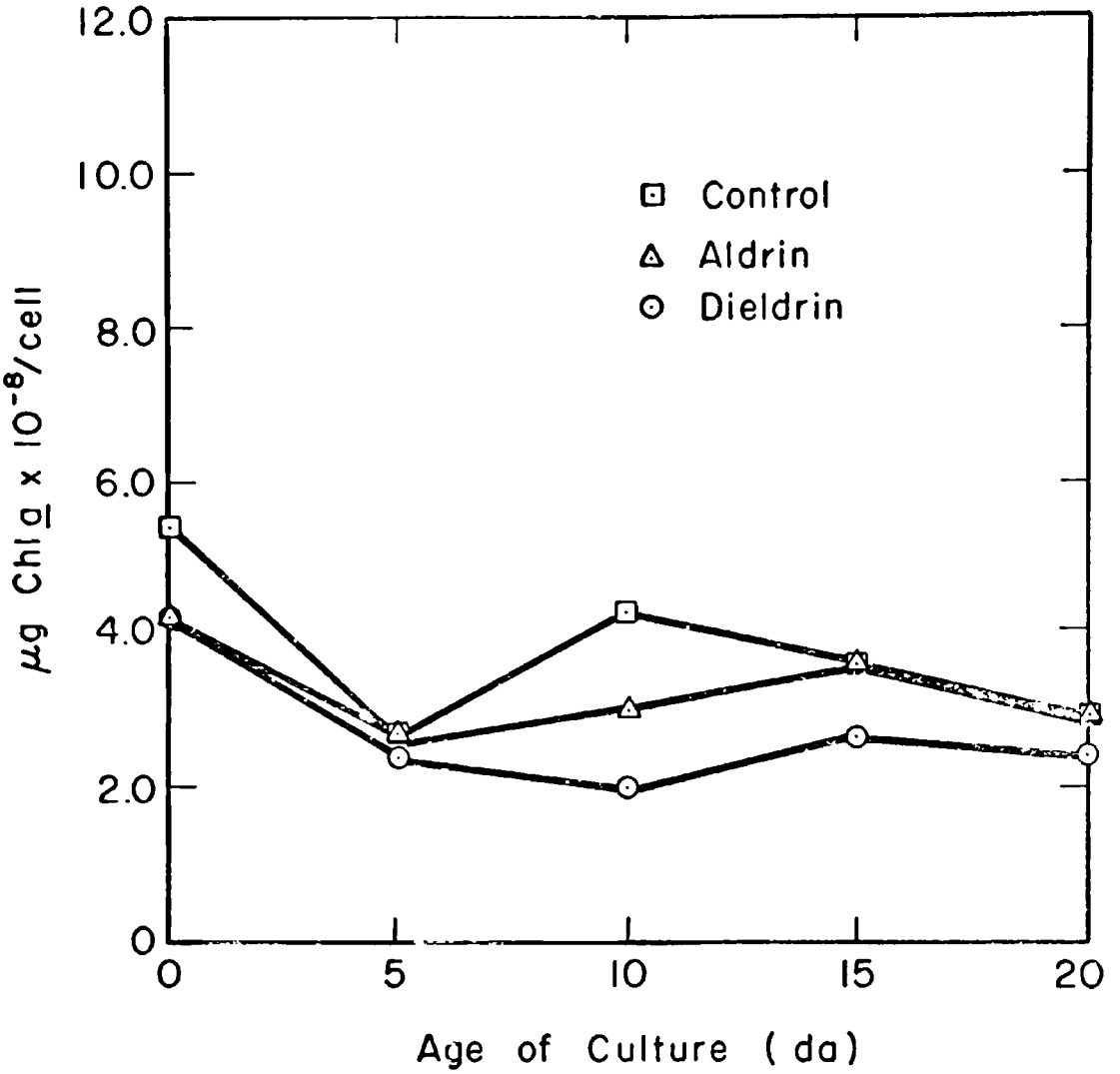

Fig. 4 -- Content of chl a per cell of A. nidulans after a single addition of pesticide. Squares represent the control; triangles, aldrin-treated cells; circles, dieldrin-treated cells.

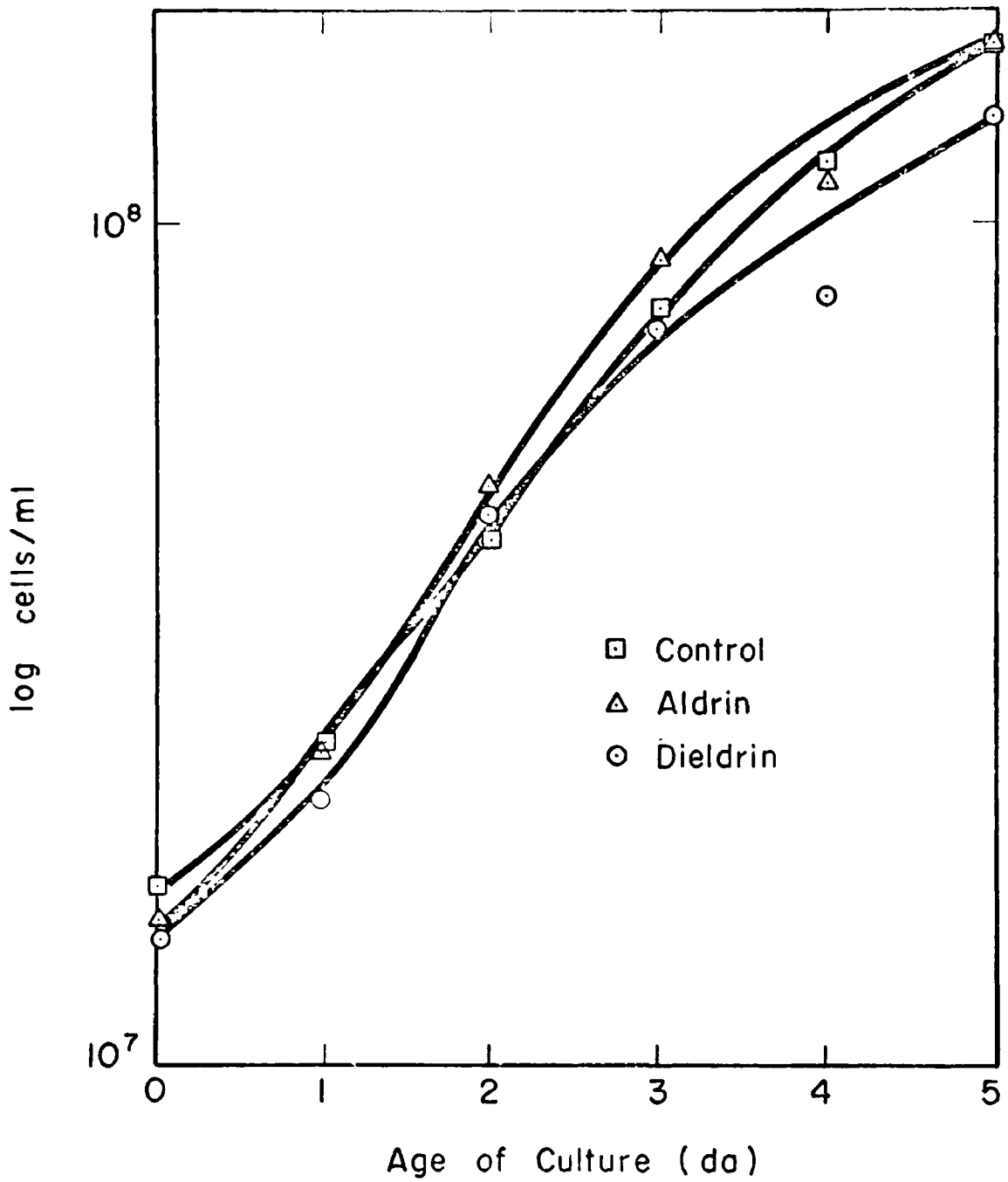

Fig. 5. -- Growth response of A. nidulans to daily pesticide addition. Squares represent the control; triangles, aldrin; circles, dieldrin.

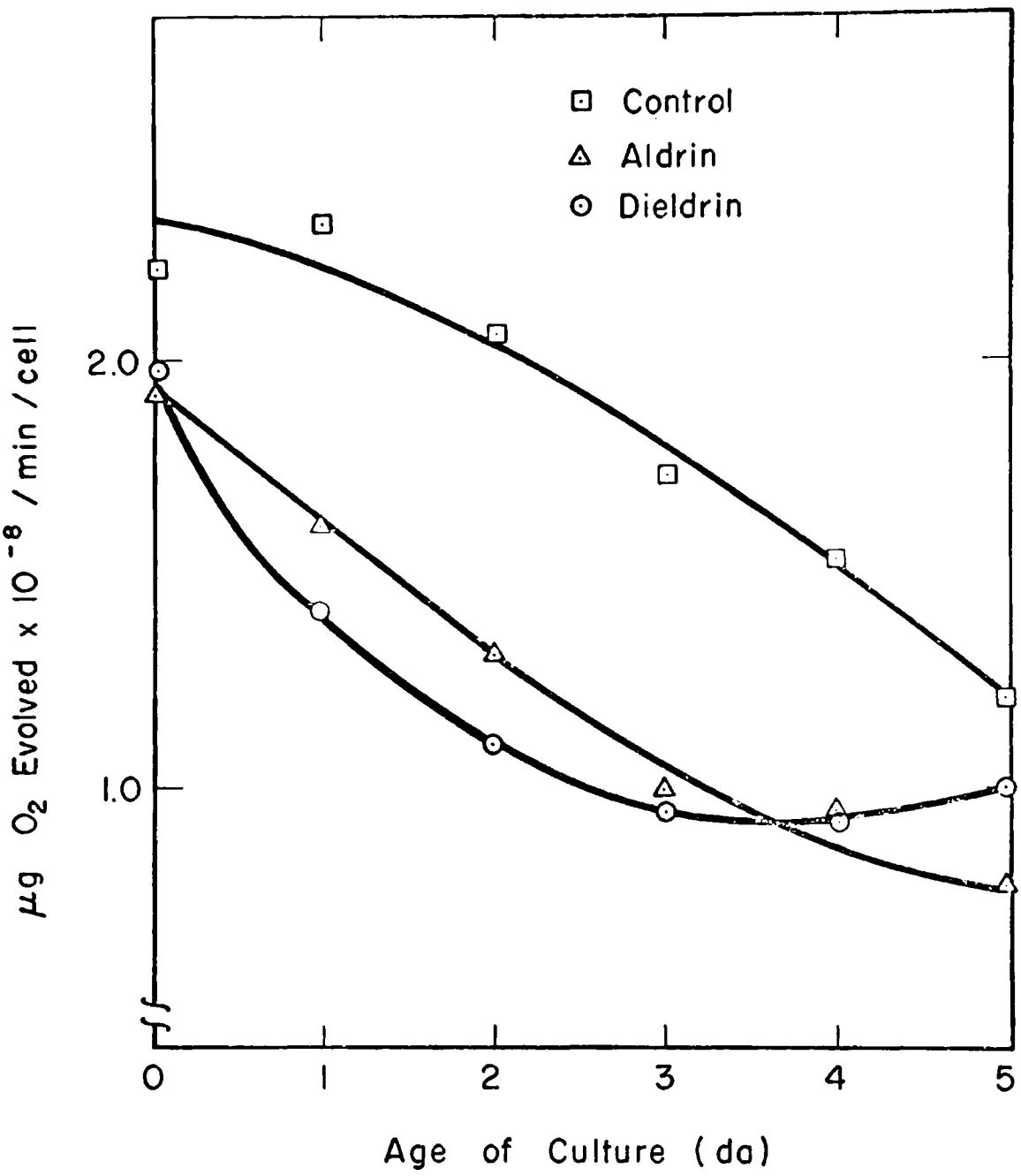

Fig. 6. -- Cellular rates of photosynthesis for A. nidulans in response to daily addition of pesticides. Squares represent the control; triangles, aldrin; circles, dieldrin.

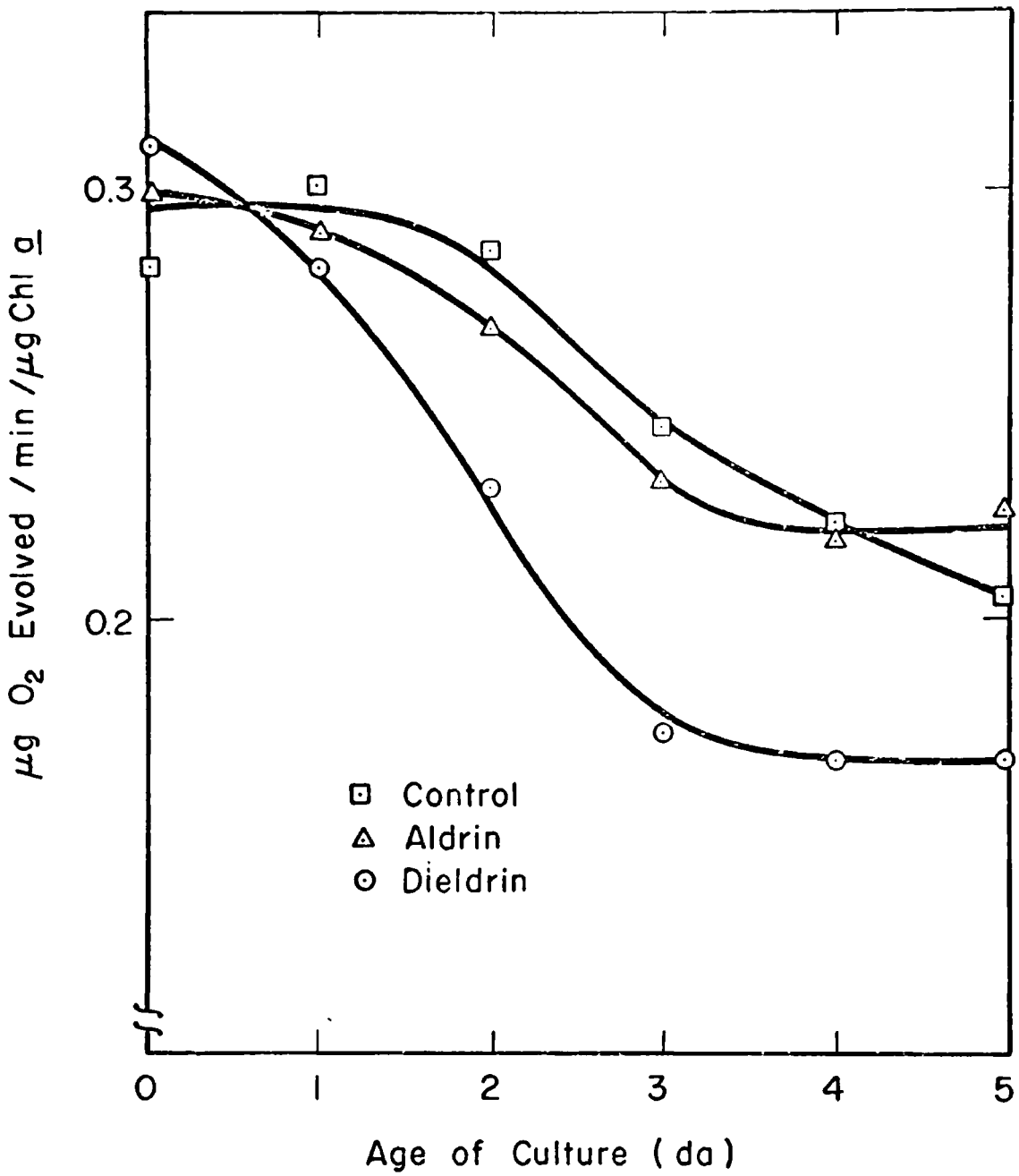

Fig. 7. -- Oxygen produced per unit chl a in response to daily additions of pesticides to A. nidulans. Squares represent the control; triangles, aldrin; circles, dieldrin.

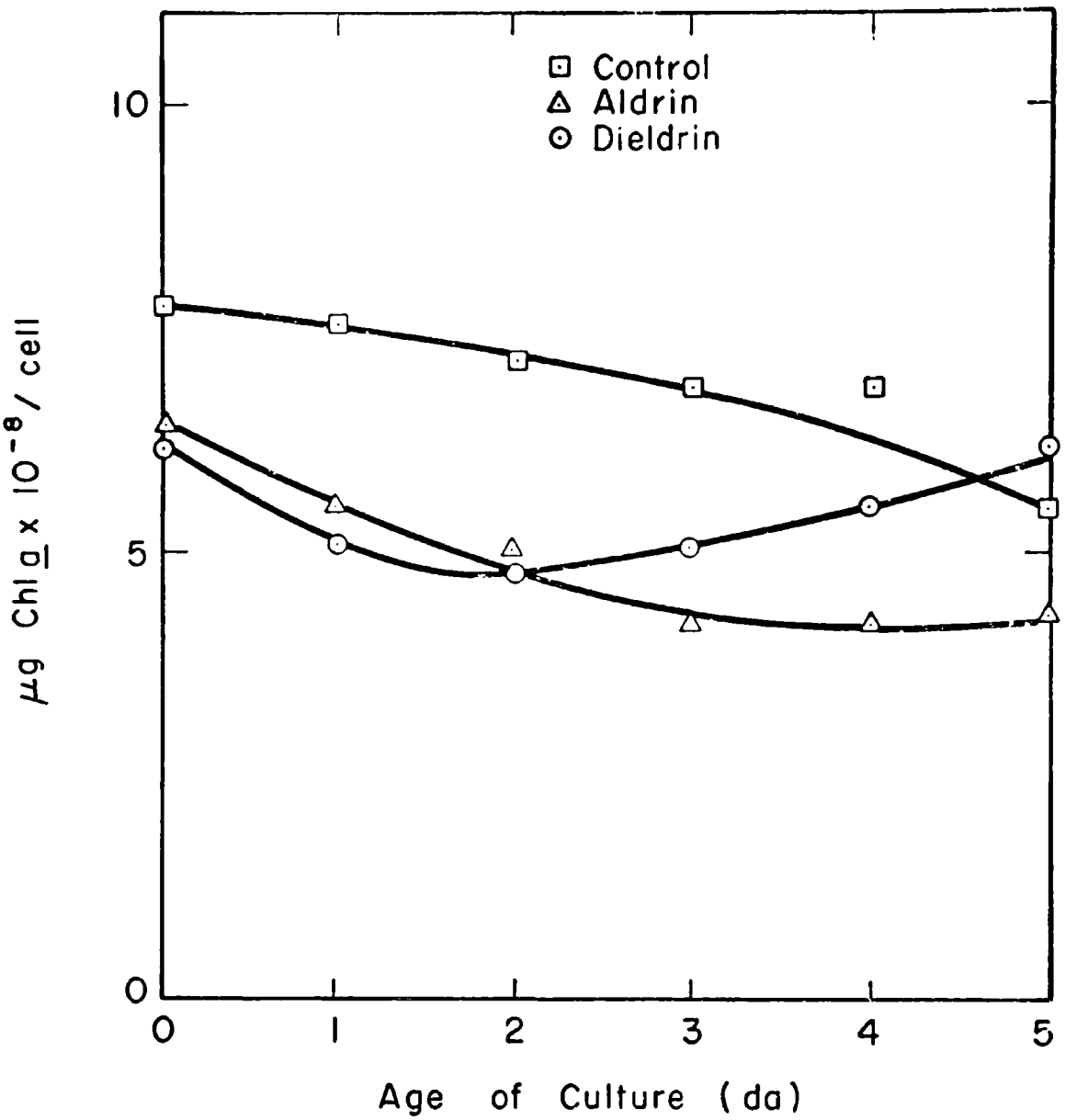

Fig. 8. -- Chl a content per cell of A. nidulans in response to daily addition of pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

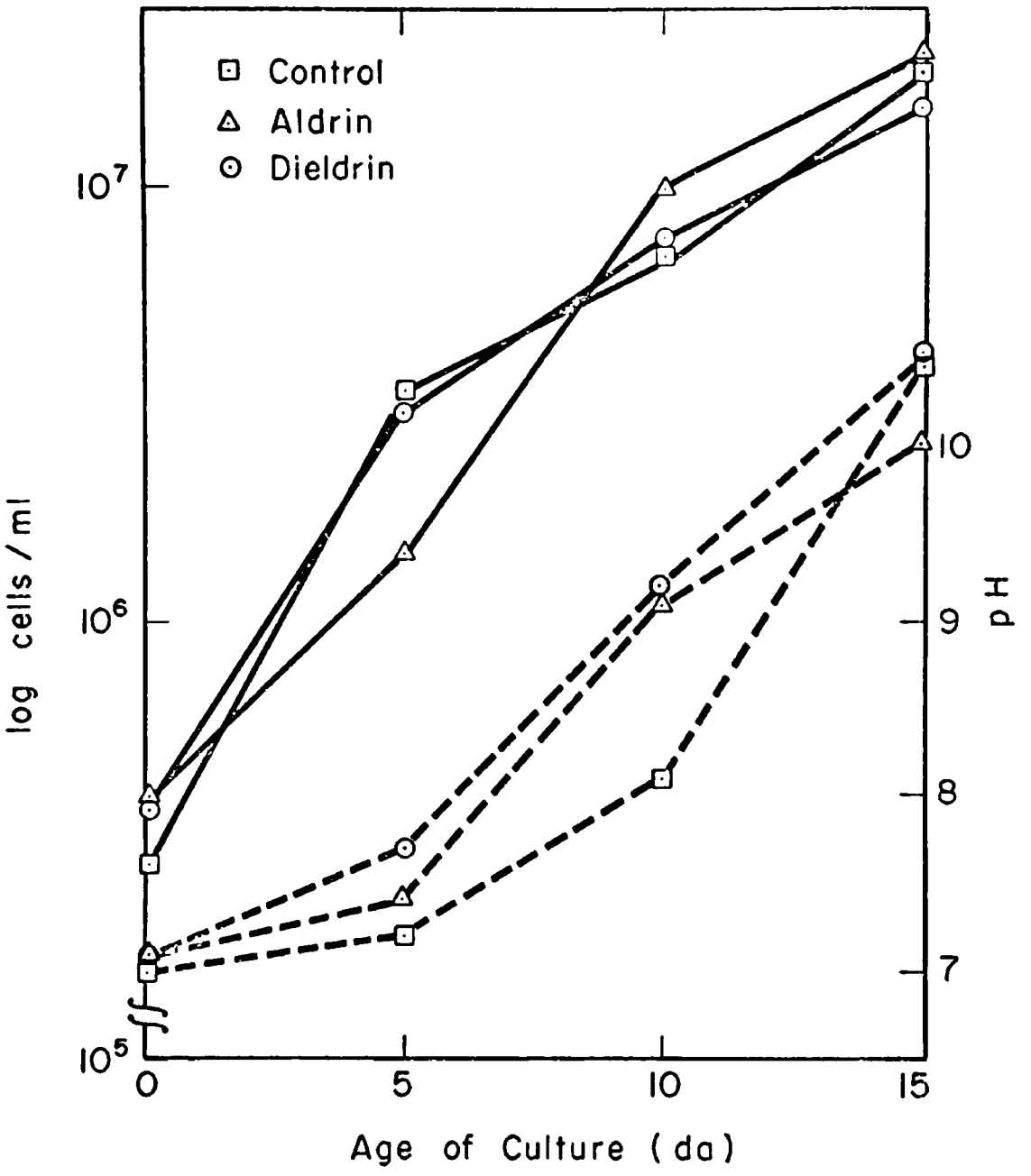

Fig. 9. -- Growth and pH changes of M. aeruginosa in response to a single addition of aldrin or dieldrin. Squares represent the control; triangles, aldrin; circles, dieldrin; solid lines, growth; dashed lines, pH.

throughout most of the growth cycle.

Dieldrin treated cells maintained slightly higher rates of photosynthesis than the control (Fig. 10) throughout the test period. After the fifth day aldrin treated cells attained rates of oxygen evolution greater than both the control and dieldrin cells. From day 10 to 15, these rates remained slightly below the control.

The amount of oxygen produced per unit of chl a was initially higher for cells treated with both pesticides (Fig. 11). During the remainder of the test period, photosynthetic rates were similar with the exception of the control on day 10.

Dieldrin treatment did not greatly alter chlorophyll content of the cells from the control over the 15-day growth cycle (Fig. 12). With the exception of day 5, chl a content of the cells was maintained at levels below the control by aldrin.

The effect of daily addition of pesticides is demonstrated in Figs. 13-16.

Both pesticides suppressed growth when compared to the control (Fig. 13).

At the beginning of the test period, both aldrin and dieldrin suppressed oxygen evolution (Fig. 14). On days 2 and 3 aldrin and dieldrin increased photosynthetic rates of the cells. By day 4 and 5 the rates returned to levels of the control.

The capacity of chl a to produce oxygen (Fig. 15) was suppressed by both pesticides but to a greater extent by aldrin. By day 4, dieldrin-treated cells attained levels comparable to the control. The oxygen evolving capacity of aldrin-treated cells remained below the control through day 4. The final value showed rates higher than the control.

Figure 16 depicts the effect of the pesticides on chlorophyll content. Aldrin increasingly inhibited the amount of chl a per cell over the 5 day test period. In dieldrin-treated cells chl a rose slightly and then fell. The chl a content of the control cells dropped precipitously and then rose to the approximate level of the dieldrin-treated cells.

Bioconcentration

The fate of a single addition of either pesticide to cultures of blue-green algae was followed at 5 day intervals for a period of 20 days (Figs. 17-20).

Relatively high concentrations of aldrin were detected in disrupted cells of A. nidulans at the beginning of the experiment (Fig. 17). There was an abrupt decrease in pesticide on day 5 which continued through 15 days. On the twentieth day the amount of pesticide detected increased to about 50% of the initial concentration from day 0.

The hexane wash, assumed to be adsorbed pesticide, showed almost no aldrin after day 5. Aldrin in the supernatant fraction remained at constant low levels for the 20-day period.

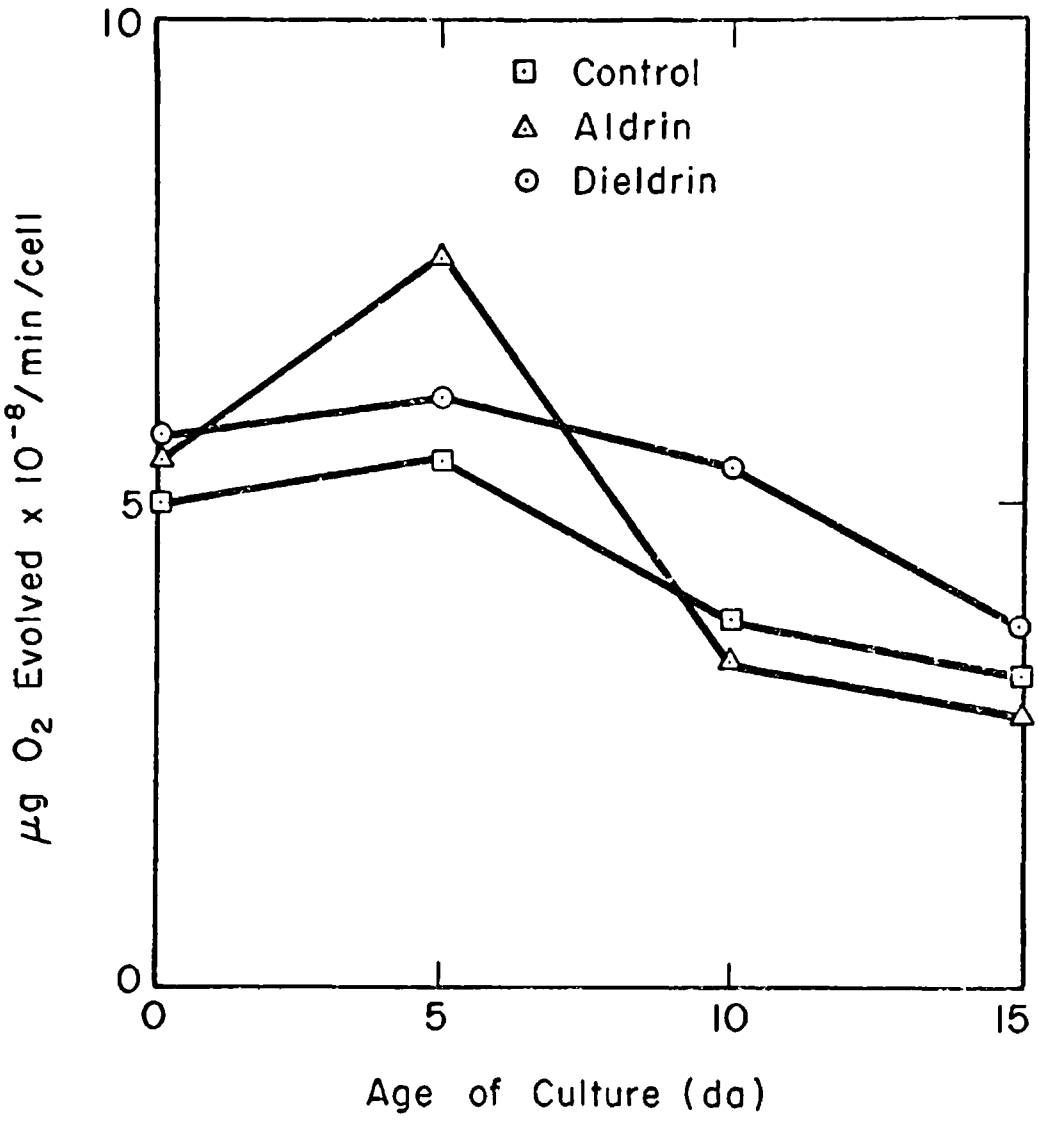

Fig. 10. -- Cellular rates of phorosynthesis by M. seruginosa in response to a single pesticide treatment. Squares represent the control; triangles, aldrin; circles, dieldrin.

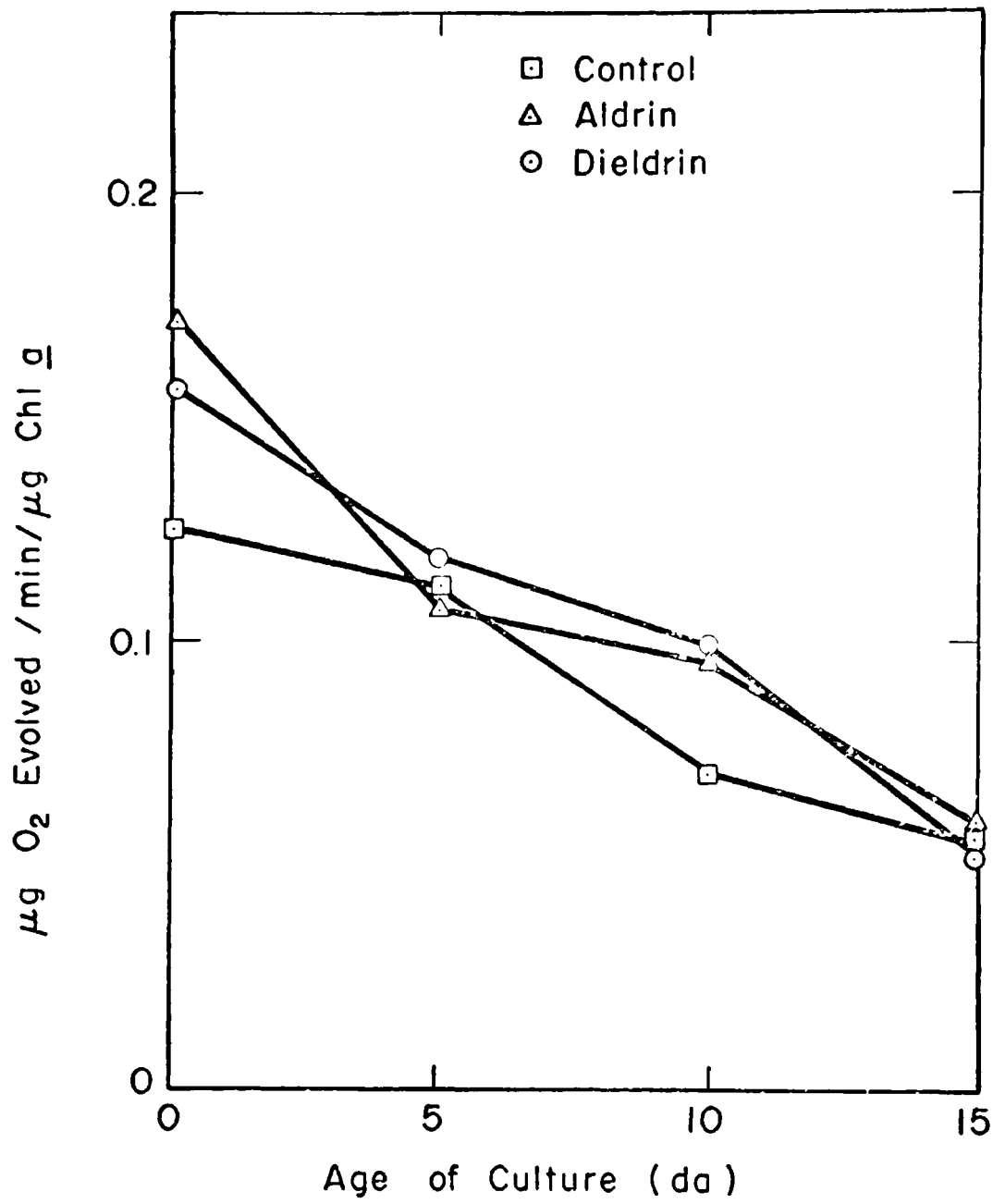

Fig. 11. -- Oxygen evolved per unit chl a in M. aeruginosa after a single treatment with pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

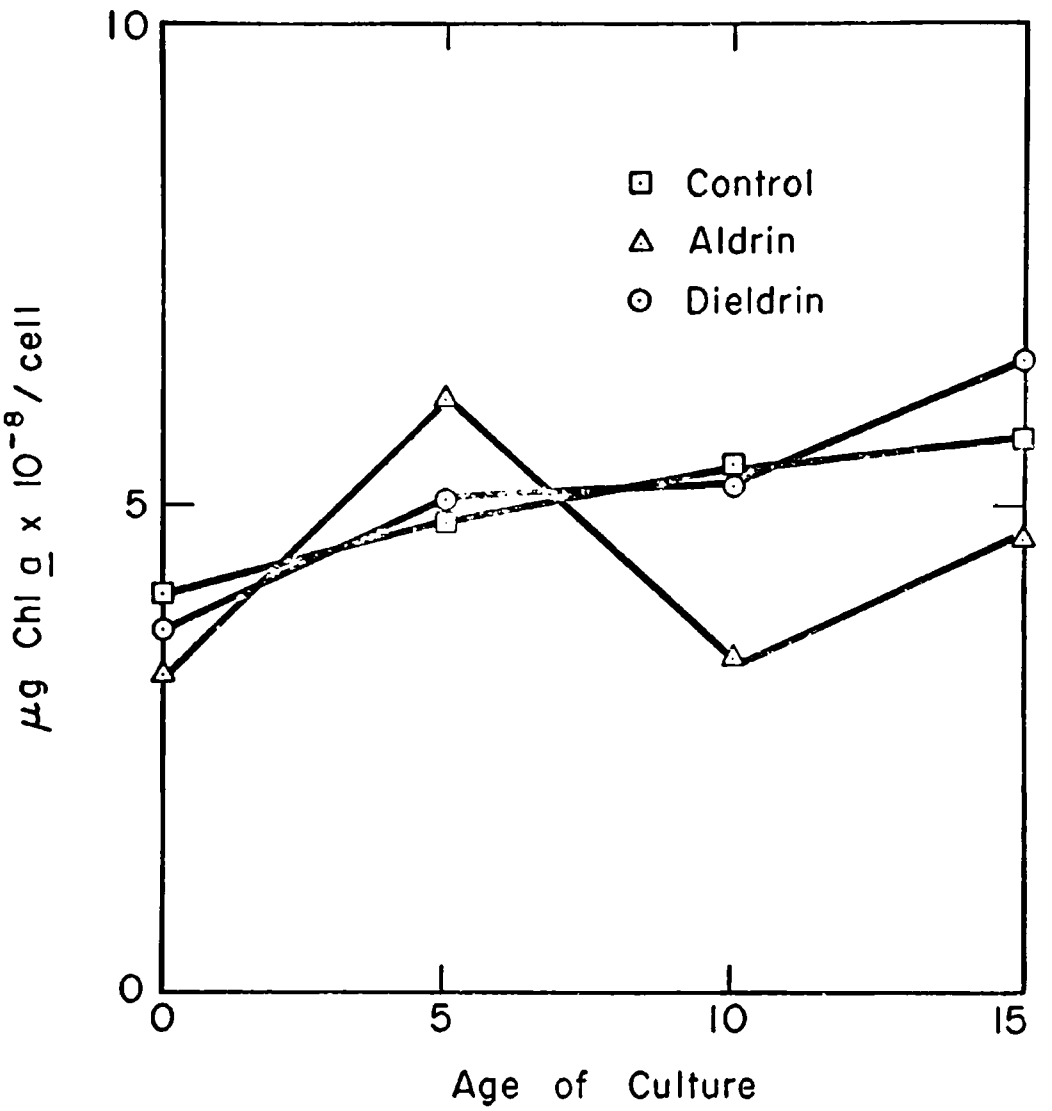

Fig. 12. -- Chl a to cell ratio in M. aeruginosa after a single addition of pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

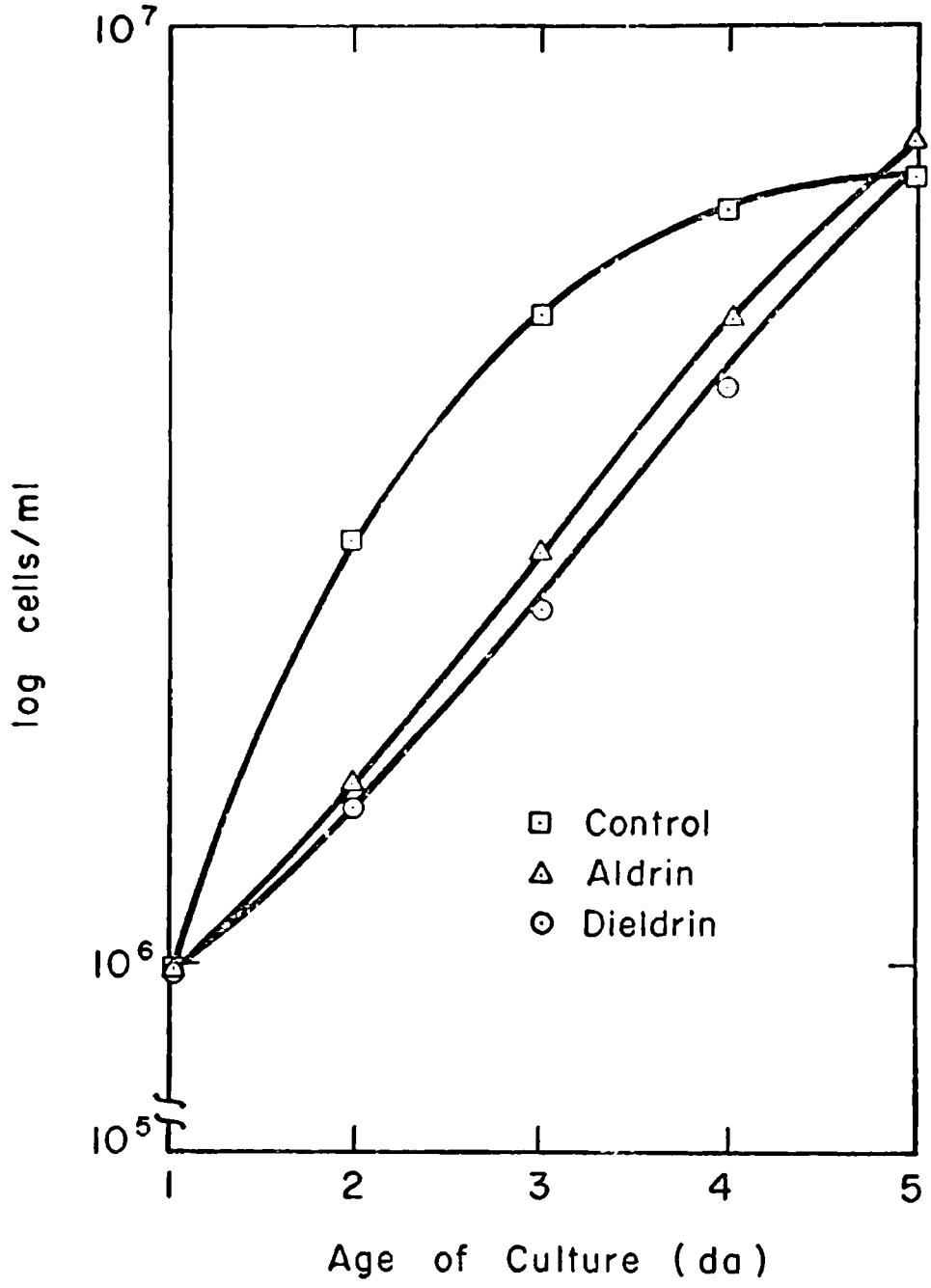

Fig. 13. -- Growth response of M. aeruginosa to daily addition of pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

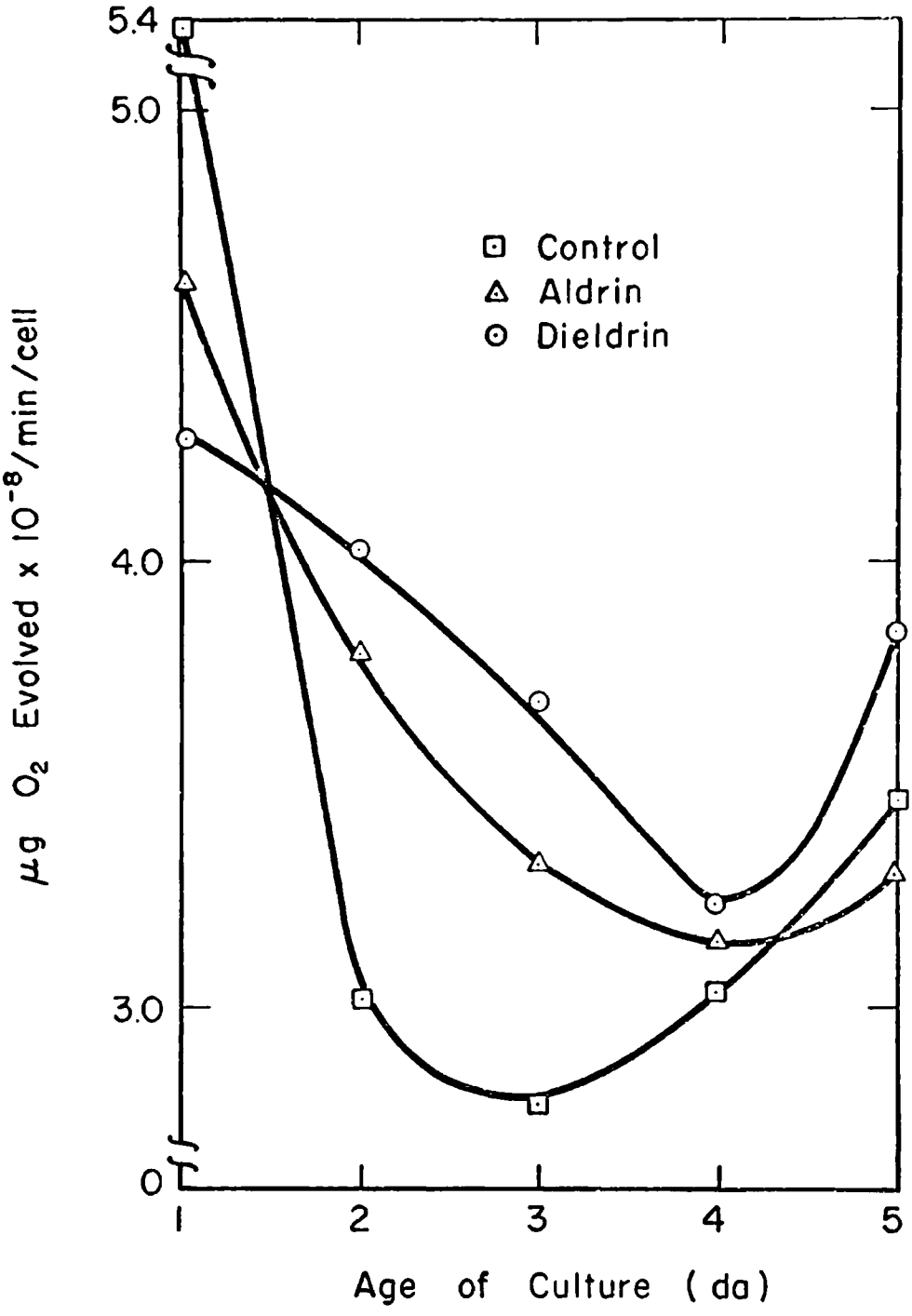

Fig. 14. -- Cellular capacity of M. aeruginosa to evolve oxygen in response to daily additions of pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

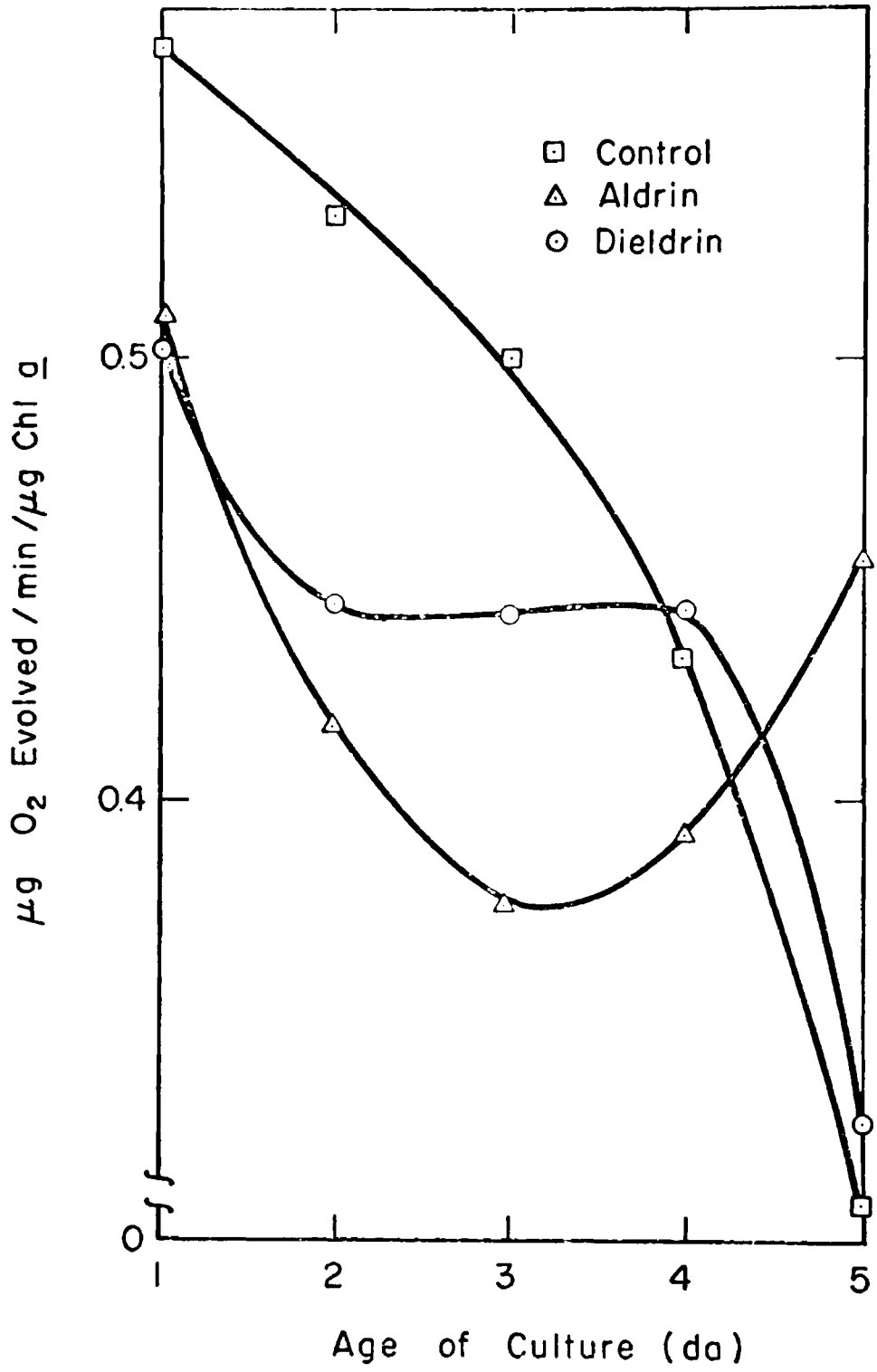

Fig. 15. -- Production of oxygen per unit chl a from M. aeruginosa in response to daily additions of pesticide. Squares represent the control; triangles, aldrin, circles, dieldrin.

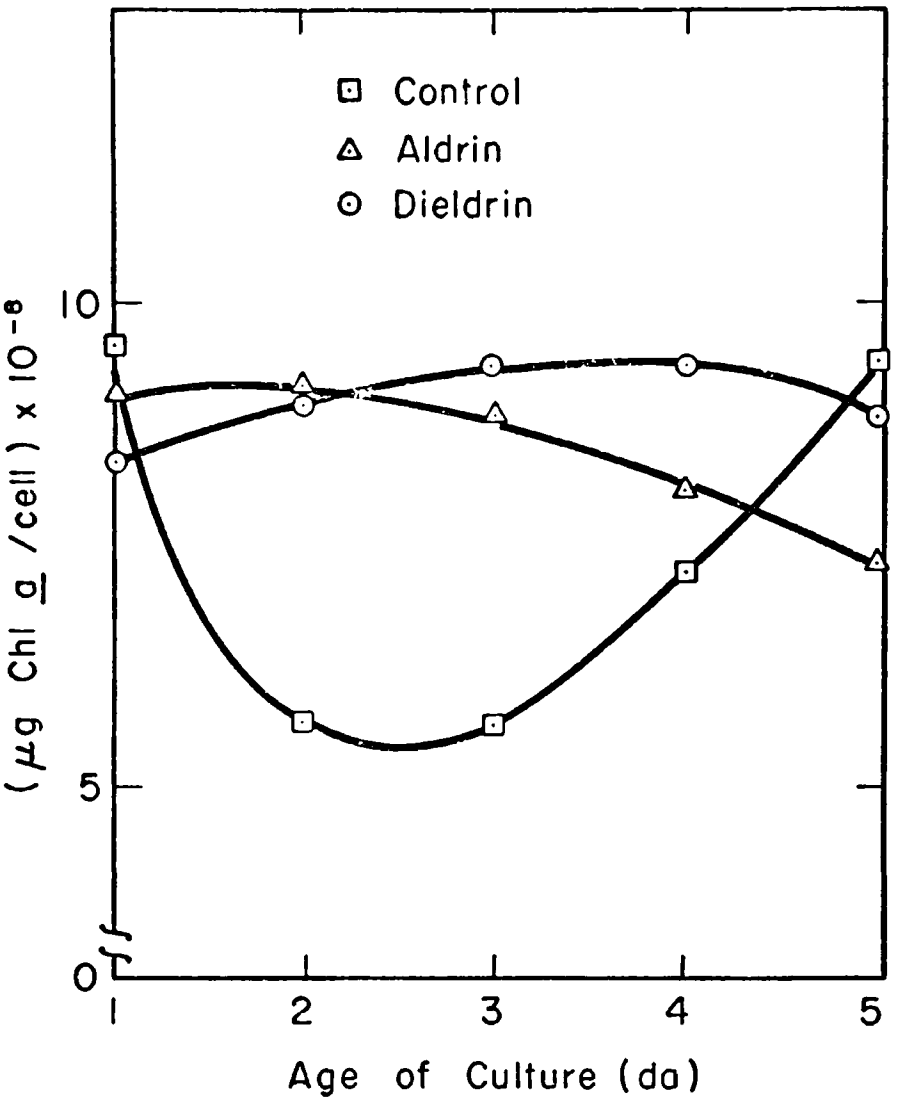

Fig. 16. -- Cell content of chl a in M. aeruginosa in response to daily additions of pesticide. Squares represent the control; triangles, aldrin; circles, dieldrin.

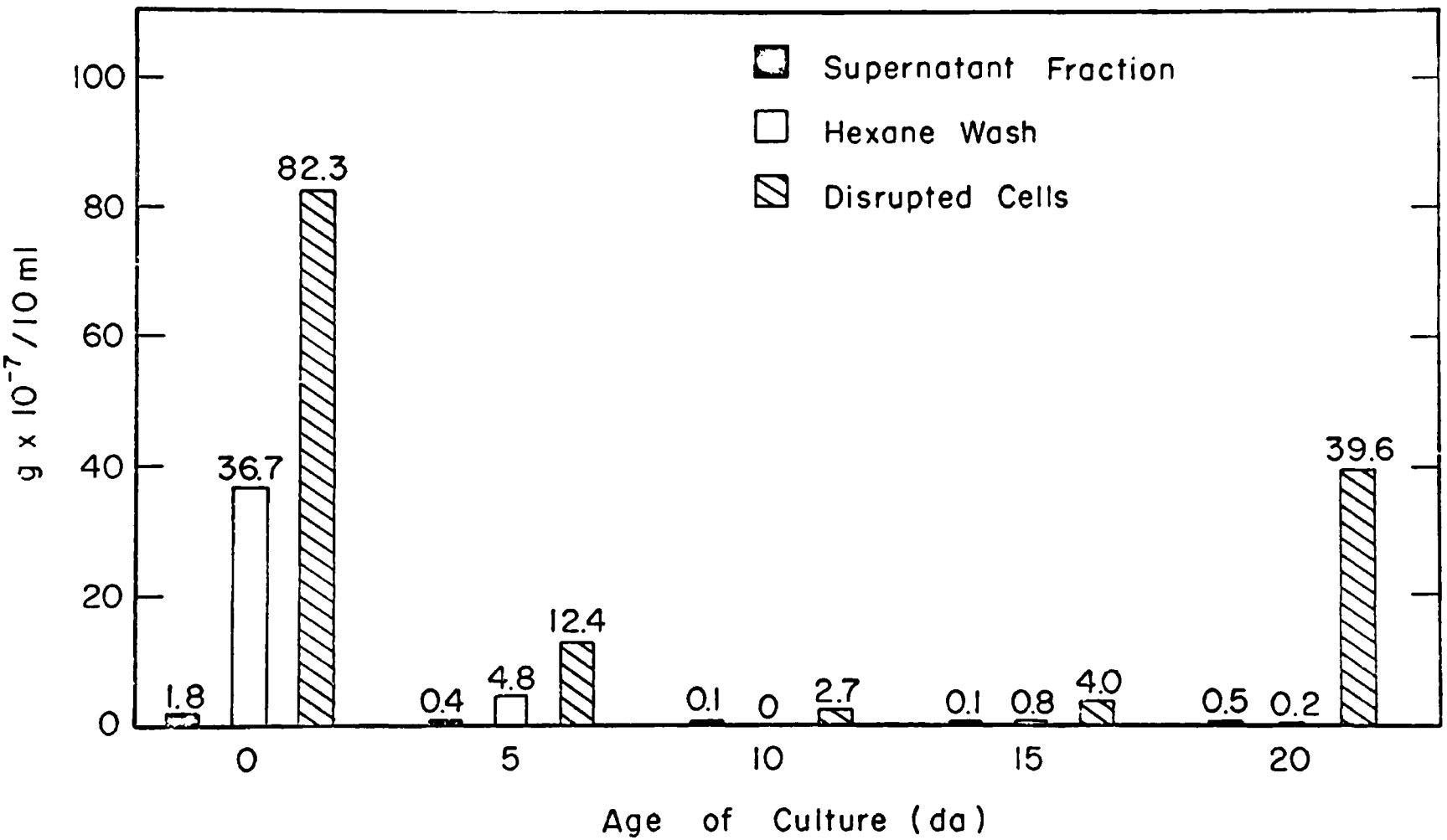

Fig. 17. -- Uptake of a single addition of aldrin by A. nidulans. Solid bars represent aldrin from the supernatant fraction; open bars, hexane wash; cross-hatched bars, disrupted cells.

For cultures of A. nidulans exposed to dieldrin, the amount of pesticide detected in the cells was relatively high initially (Fig. 18). From 1-15 days the amount detected in the cells was low. On day 20, as with aldrin, the amount increased. Unlike the aldrin-treated cells, the presence of dieldrin in the hexane wash increased from an initial low to high concentrations (day 10) and then decreased. The amount of pesticide in the supernatant fraction remained at constantly low levels.

The amount of aldrin recovered from the culture of M. aeruginosa showed a decreasing trend over the 20-day period (Fig. 19). The hexane wash demonstrated a similar trend. However, the amount adsorbed to the cells was much greater initially than absorbed in the cells. This is in contrast to cells of A. nidulans treated in a similar manner (cf. Fig. 17). The supernatant wash again did not contain high concentrations of the pesticide.

Dieldrin associated with cells of M. aeruginosa dropped slightly at 5 days and then remained constant (Fig. 20). The hexane wash reached a maximum concentration of the fifth day, thereafter decreasing. The low concentrations of dieldrin in the supernatant fraction showed little variation.

In Table 2 is presented pesticide recovery values from the preceeding experiments. The controls gave consistently lower per cent recovery than the cell culture. Aldrin recovery was generally lower than dieldrin. At higher temperatures the loss of pesticide was greater than at ambient temperature. Very little of the pesticides adsorbed to the culture vessel: less than 1% for aldrin and about 10% for dieldrin. The recovery amounts obtained are generally lower than acceptable. This may have been due to codistillation of the pesticides with water. The higher recovery values for the cultures over the controls suggests that the cells provide surfaces for the absorption of the pesticides (Sodergren, 1968).

Electron Microscopy

The results of the cytological study on A. nidulans and M. aeruginosa are presented in Figs. 21-25.

The identification of altered structure due to pesticide treatment was of primary importance to this study. Any localized sites of activity should aid in the elucidation of the pesticide interference mechanisms.

Electron micrographs of A. nidulans depicting representative sections from pesticide treatments and the control are shown in Fig. 21. All contain similar structures with no apparent changes due to treatment with pesticides. The rugose nature of the cell wall in Fig. 21b is not limited to the aldrin treated cells but is generally found among all cell-types. Figure 21c shows most of the structures found in actively growing cultures of A. nidulans. As can be seen more readily in Fig. 23a, the cell wall (cw) is composed of 4 layers. The L I layer is electron transparent lying just outside the plasma membrane (pm). The L II layer is an electron dense area followed by another electron transparent layer (L III). The outer tripartite membranous structure is L IV.

The membranous structures within the cytoplasm forming concentric shells are the

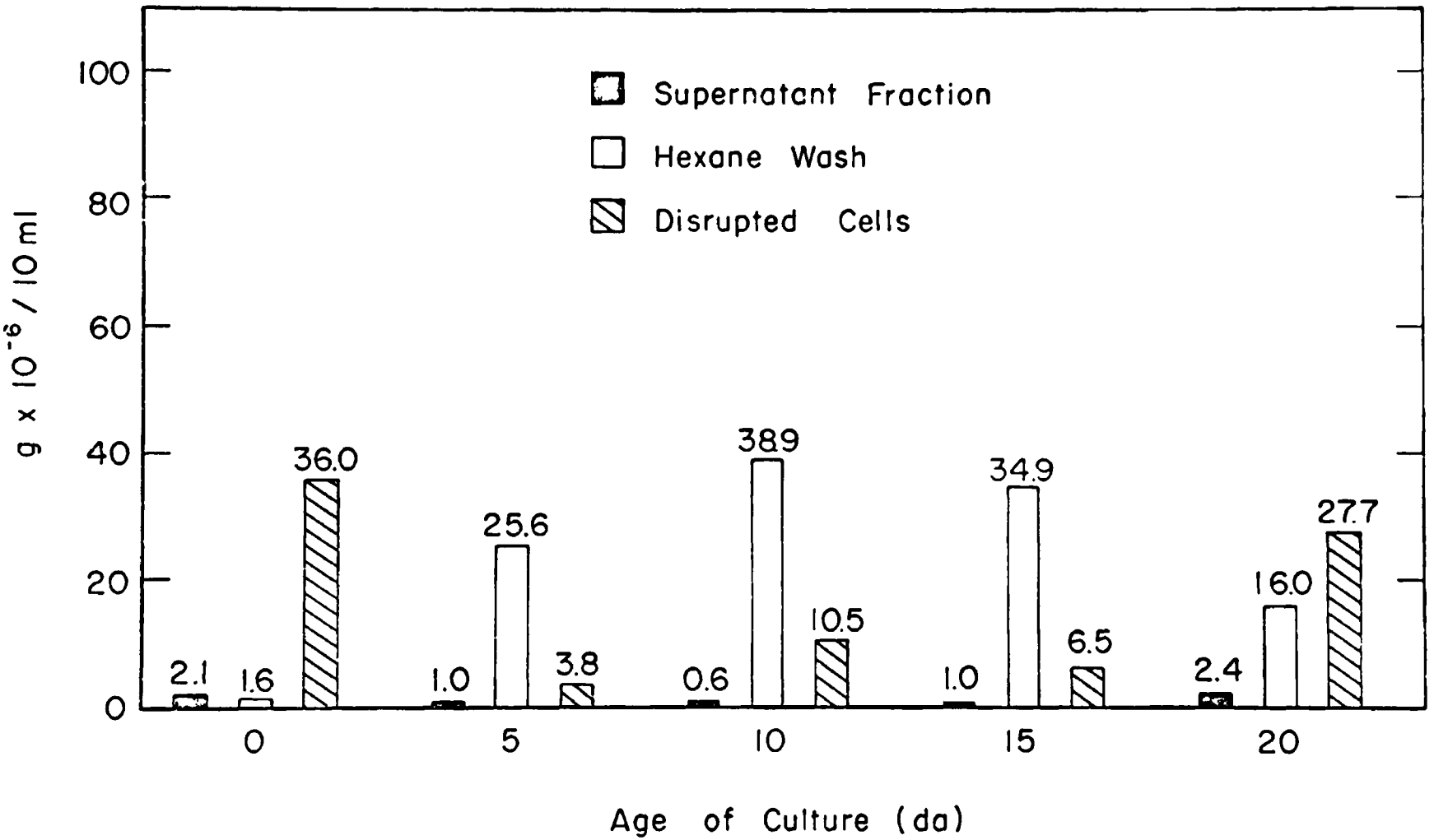

Fig. 18. -- Uptake of a single addition of dieldrin by A. nidulans. Solid bars represent dieldrin in the supernatant fraction; open bars, hexane wash; cross-hatched bars, disrupted cells.

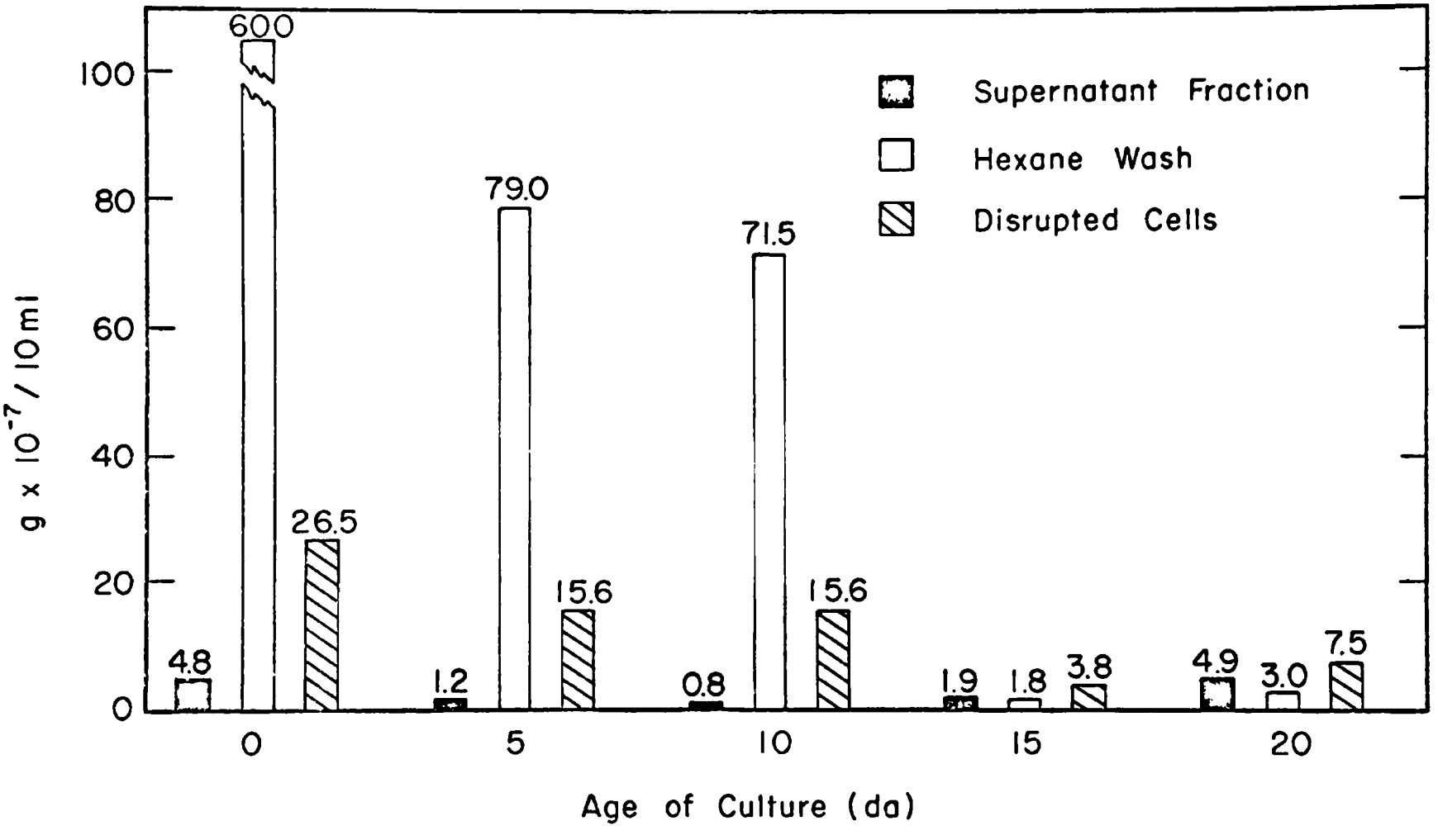

Fig. 19. -- Uptake of a single addition of aldrin by M. aeruginosa. Solid bars represent aldrin in the supernatant fraction; open bars, hexane wash; cross-hatched bars, disrupted cells.

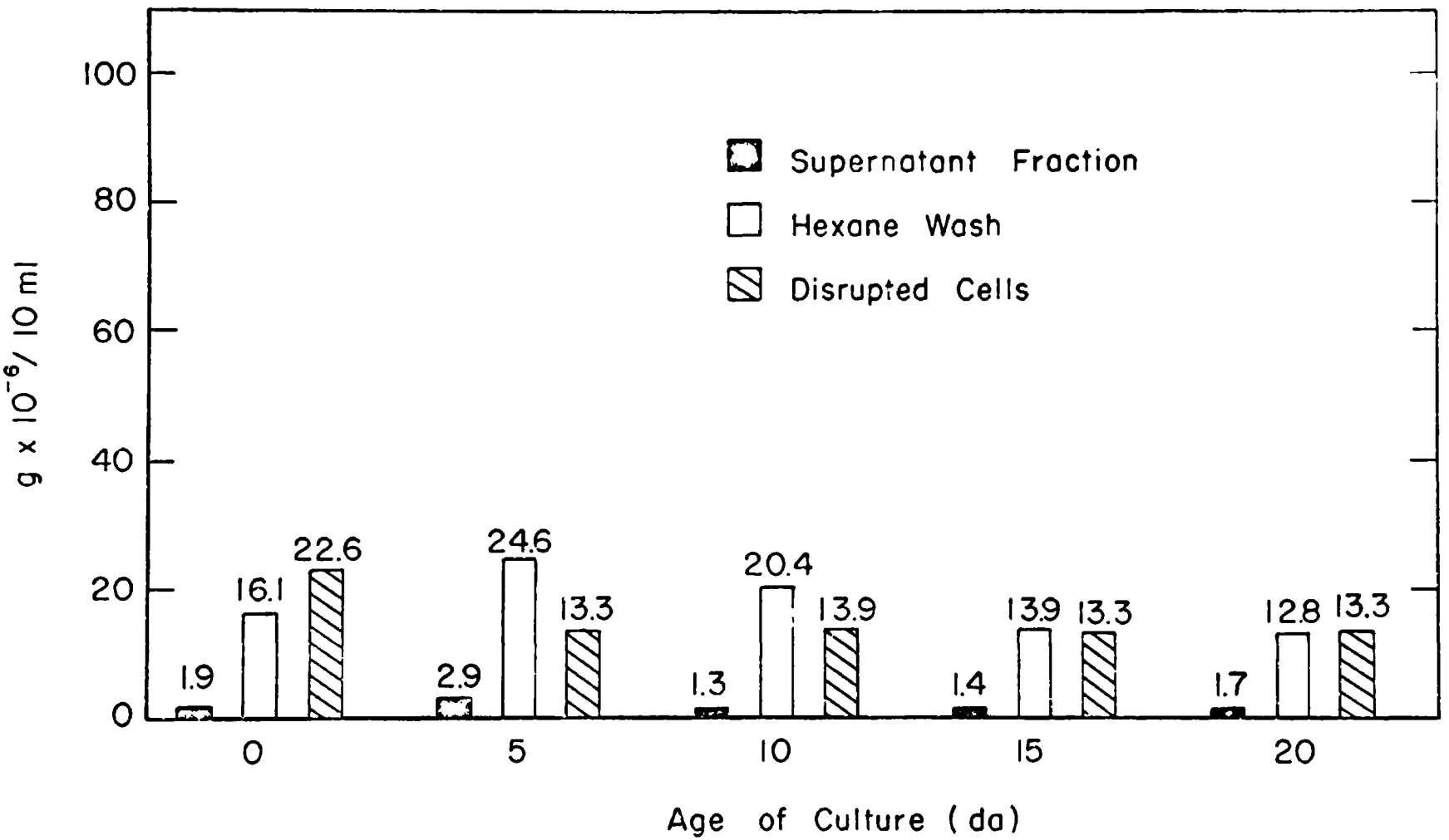

Fig. 20. -- Uptake of a single addition of dieldrin by M. aeruginosa. Solid bars represent dieldrin from the supernatant fraction; open bars, the hexane wash; cross-hatched bars, disrupted cells.

TABLE 2.--Per cent recovery of pesticides from the culture.[a]

Description		Per cent recovery[b] Age of culture (da) 0	5	10	15	20	flask rinse
Aldrin	Control[c]	25.6	0.9	--[d]	9.2	0.09	0.1
	A. nidulans	18.5	2.7	0.4	0.8	6.2	--
	Control	27.7	7.6	2.8	0.7	0.1	0.2
	M. aeruginosa	97.0	14.6	13.4	1.2	2.4	--
Dieldrin	Control	39.6	2.4	16.7	13.9	4.3	10.8
	A. nidulans	61.1	46.7	77.0	69.8	70.8	--
	Control	43.7	16.7	25.0	12.1	4.4	7.29
	M. aeruginosa	62.3	62.8	54.7	44.2	42.7	--

[a]Wash plus supernatant fraction plus disrupted cells.
[b]Based on the amount added to the culture.
[c]Controls contained pesticide but no cells; maintained at temperature of the cells.
[d]Not determined.

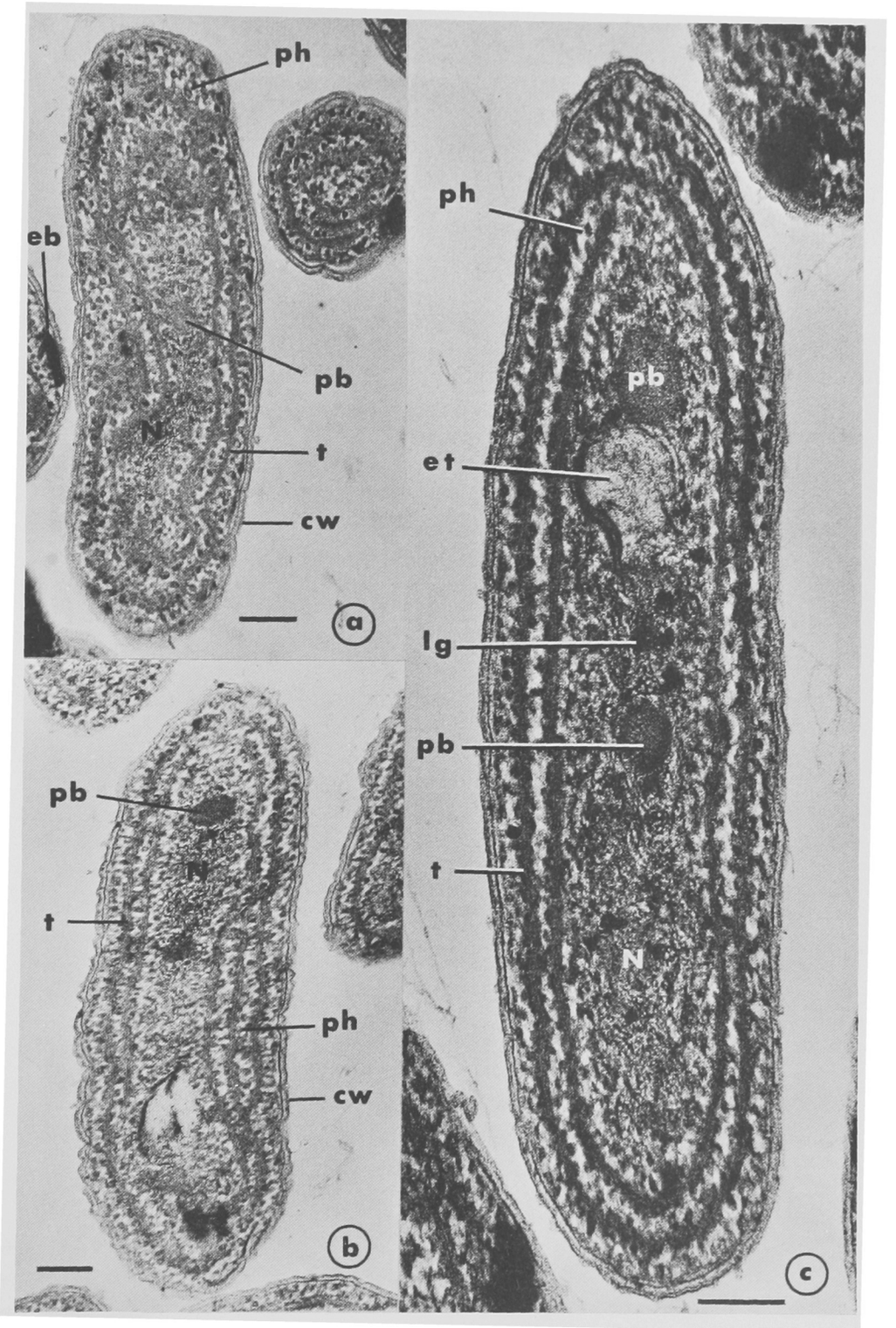

Fig. 21. -- Ultrastructure of A. nidulans after daily treatment with pesticide for 5 days. Control cells, a; aldrin-treated cells, b; dieldrin-treated cells, c. The cell wall (cw), the thylokoids (t), phycobilisomes (ph), and nuclear material (N) can be seen. In addition, several inclusions are apparent: polyhedral body (pb), electron dense elongated body (eb), libid globules (lg) and electron transparent body (et). Fixed with gluteraldehyde-osmium; embedded in Maraglas. Bar equals 0.2 μm.

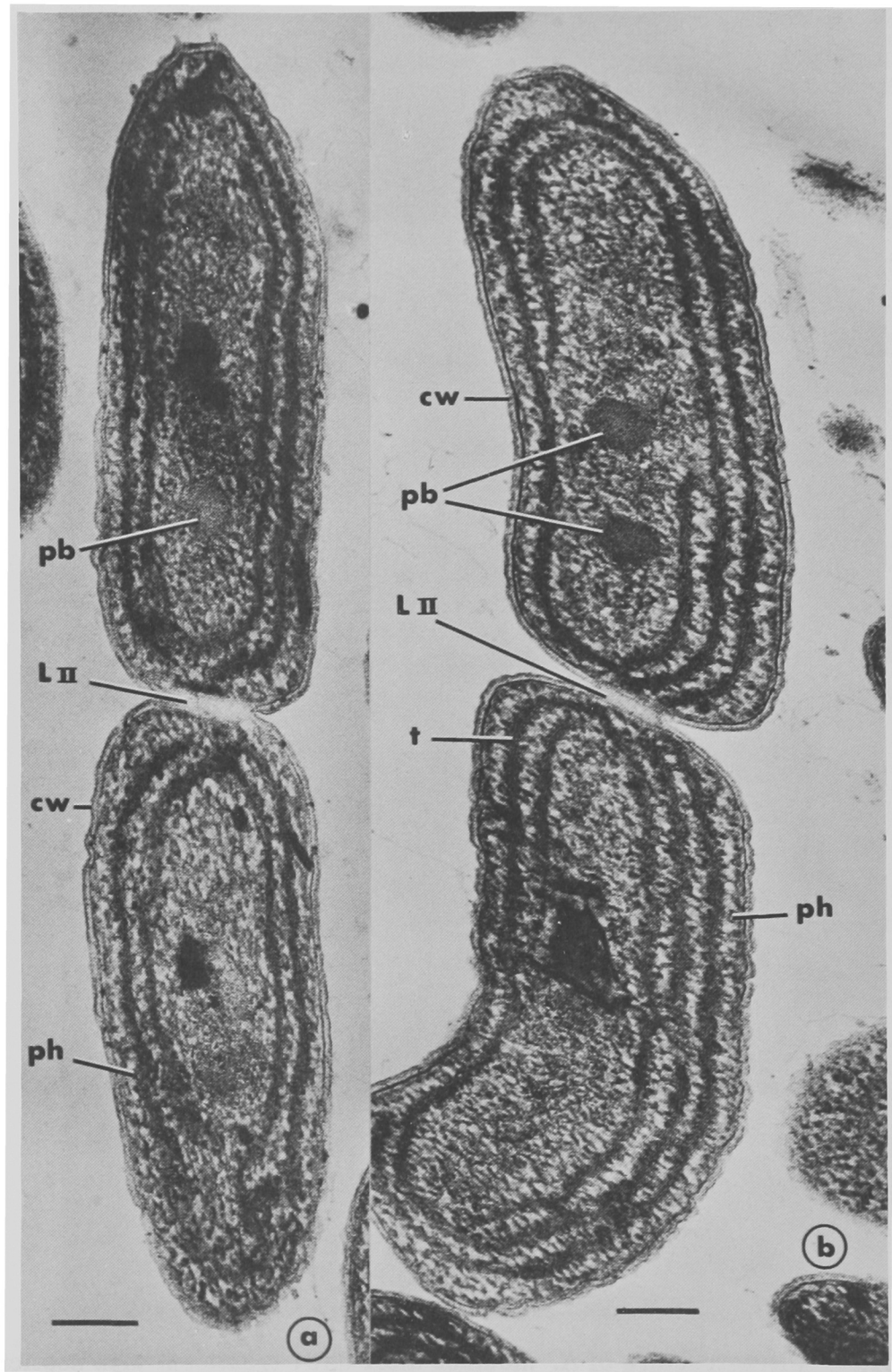

Fig. 22. -- Ultrastructure of dividing cells of A. nidulans treated with dieldrin. Early stage of cell division, a; later stage, b. The importance of the L II layer of the cell wall (cw) in the division process is apparent. The thylokoids (t), polyhedral bodies (pb) and phycobilisomes (ph) can also be seen. Fixed with glutaraldehyde-osmium; embedded in Maraglas. Bar equals 0.2 μm.

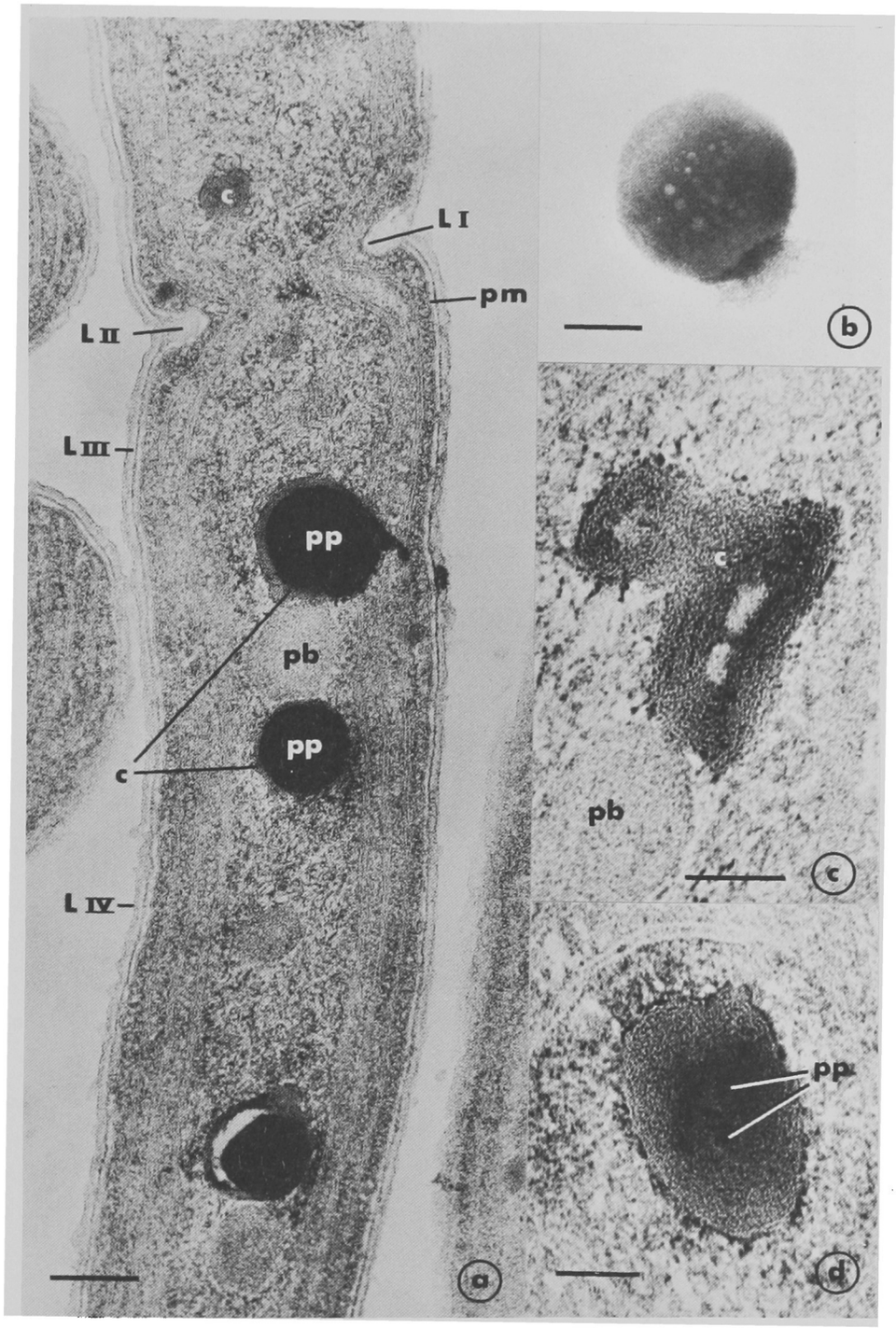

Fig. 23. -- Ultrastructure of A. nidulans. Section from a 42-day old culture showing the L II layer of a developing septum, cyanophycin granules (c), polyphosphate bodies (pp) and polyhedral bodies (pb), a. Other layers of the cell wall, L I, L III and L IV as well as the plasma membrane can also be seen. Bar equals 0.2 μm. Late stage of a developing polyphosphate granule, b. Bar equals 0.1 μm. Cyanophycin granule (c) in a mesosomal configuration, c. Bar equals 0.1 μm. Cyanophycin granules (c) showing polyphosphate decomposition, d. Bar equals 0.1 μm. Glutaraldehyde-osmium fixation; embedded in Maraglas.

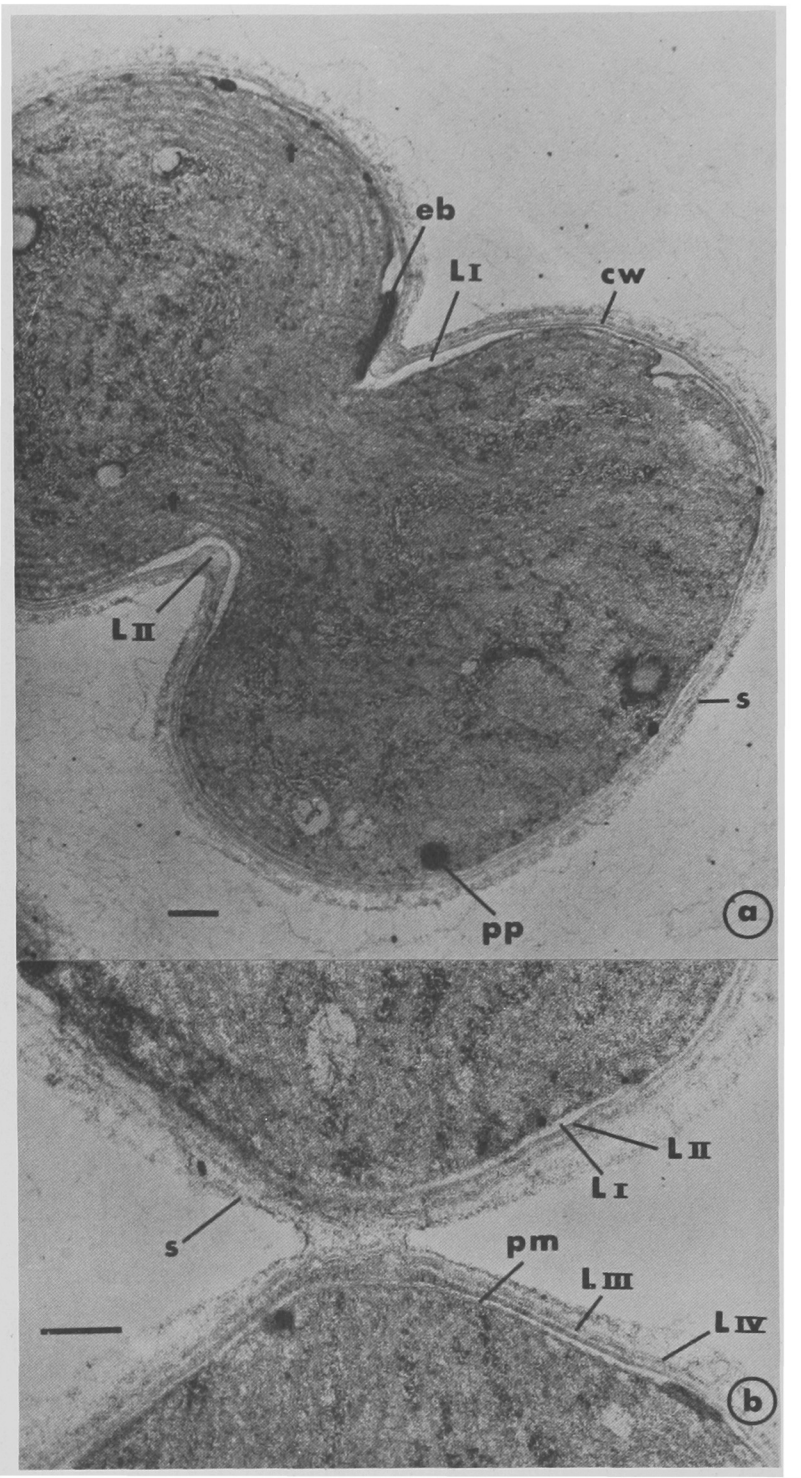

Fig. 24. -- Ultrastructure of M. aeruginosa from the daily addition of pesticide study: a, dividing cell from the control; b, recently divided cell from the control. The sheath (s), layers (L I, L II, L III, L IV) of the cell wall (cw), thylakoids (t), nucleoplasm (N), polyphosphate granules (pp) and an electron dense elongated body (eb) may be seen. Gluteraldehyde-osmium fixation; embedded in Maraglas. Bar equals 0.2 μm.

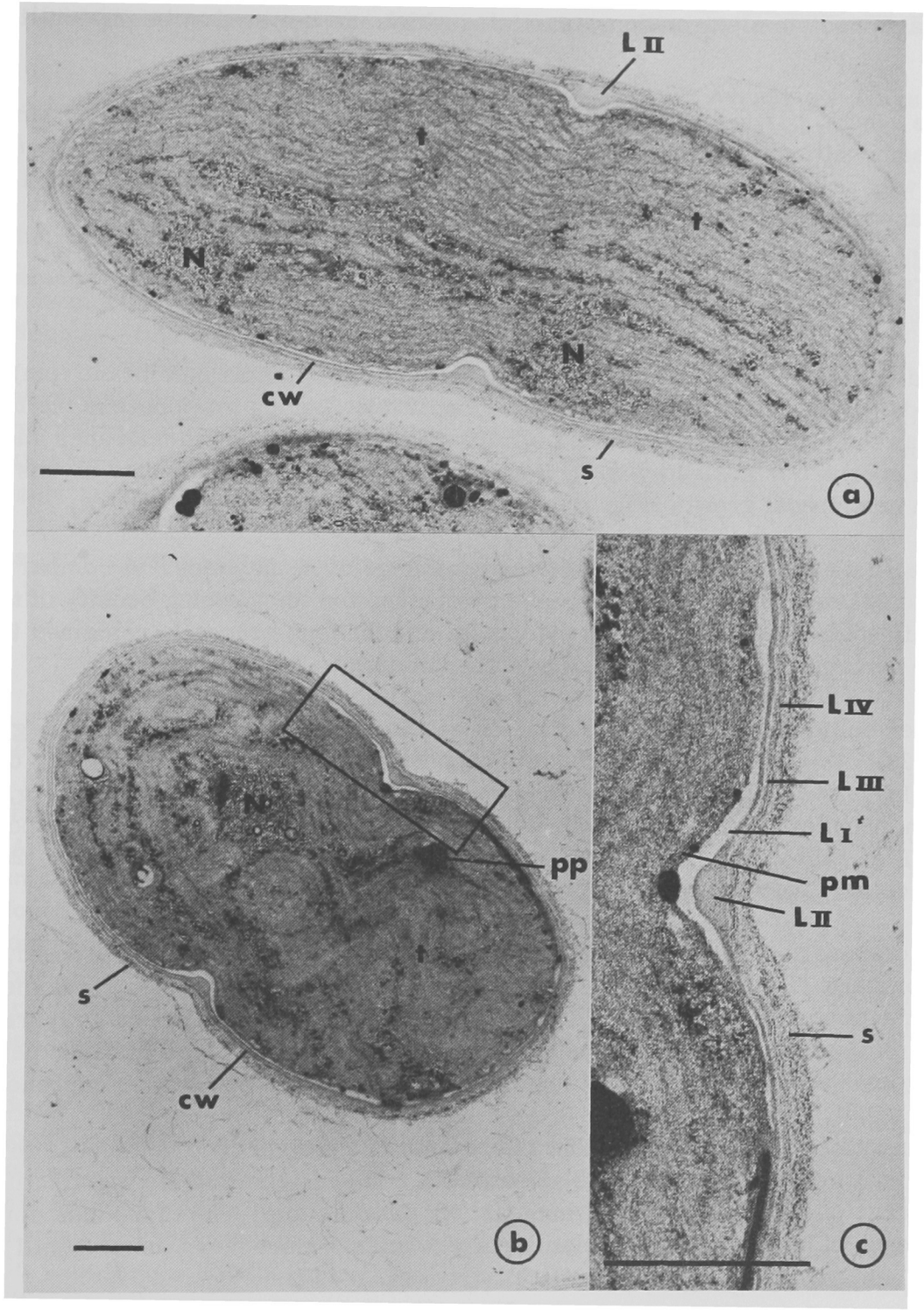

Fig. 25. -- Ultrastructure of M. aeruginosa: a, aldrin treated cell; b, cell treated with dieldrin in early stages of division; c, higher magnification of the rectangular area of b. Also the sheath (s), layers L I, L II, L III, L IV) of the cell wall (cw), plasma membrane (pm), polyohpsphate granules (pp), and nucleoplasm (N) can be seen. Glutataldehyde-osmium fixation; embedded in Maraglas. Bar equals 0.4 μm.

thylakoids (t) wherein is contained the photosynthetic apparatus. Associated with the thylakoids are medium electron dense particles which are believed to be phycobilisomes (ph).

In the central portion of the cell is located the nucleoplasm (N). Also within this area are small osmophilic bodies known as lipid globules (lg). The large medium electron dense structures with a granular appearance are polyhedral bodies (pb). Occasionally a large electron transparent body (et) can be discerned.

Figure 22 shows cells of A. nidulans treated with dieldrin undergoing normal cell division.

Cell division arises as a result of invagination of the plasma membrane (pm) followed by centripedal proliferation of the L II layer (Fig. 23a). During this process the thylakoids (t) as well as the cytoplasm are pinched off. Once the invaginating membranes anastamose, the division aperture is sealed by the L II layer (Fig. 22a). Then the outer layers (L III, L IV) begin centripedal growth (Fig. 22b) until two daughter cells are formed.

There was some variability in the electron image of A. nidulans. A comparison of Figs. 22 and 23a demonstrates that only the cytoplasm and the staining density of the thylakoids was affected. The cell wall and cytoplasmic inclusions generally remained intact. There is no certainty as to the cause of this phenomenon to date.

In older cultures structures occur which are not normally found in actively growing cells (Fig. 23). Polyphosphate granules (pp) appear as large extremely electron dense inclusions (Fig. 23a) unless photographically enlarged and printed separately (Fig. 23b). Cyanophycin granules (c) appear as membranous elaborations often surrounding polyphosphate inclusions (Fig. 23a). They variously appear as mesosomal-like elements (Fig. 23c) or as a tightly coiled structure possibly containing polyphosphate residues.

When cells of A. nidulans are fixed with permanganate, the image presented is quite different (compare Fig. 23a and 26a). The most prominent alteration occurs at the cell wall. Both the plasma membrane and L IV layer remained intact; the other layers of the cell wall were digested by the treatment. Unlike the image of osmium fixation, permanganate strongly suggests a similarity in structure between L IV and the plasma membrane.

Treatment of M. aeruginosa with either aldrin or dieldrin does not appear to have affected the integrity of the cells (Figs. 24 and 25). These representative cells all show similar patterns of thylakoidal (t) and nuclear (N) distribution. The thylakoids demonstrate various patterns of concentric shells and layered stacks. Moreover, the cell wall (cw) was not altered.

The cell envelope of M. aeruginosa exhibits the L I, L II, L III and L IV layers previously described for A. nidulans (Fig. 25c). Note that layer L I is particularly thick at the septum. In addition to these components, there is an external fibrous sheath.

Cell division occurs as a result of the invagination of the plasma membrane (pm)

followed by centripedal growth of L II (Fig. 24a, b). Unlike A. nidulans, M. aeruginosa appears to undergo more of a constrictive mode of cell division (Fig. 24a).

Polyphosphate granules (pp) were found routinely in M. aeruginosa (Figs. 24 and 25b). Occasionally they were found enclosed by cyanophycin granular material (Fig. 24a). An electron dense elongate body was also occasionally found in both A. nidulans (Fig. 21a), and M. aeruginosa (Fig. 24a).

When cells of M. aeruginosa are fixed with permanganate they show an altered cell structure (Fig. 26b). The thylakoids (t) appear more electron dense while the nuclear area (N) is more electron transparent. The sheath (s) appears to have a distinct boundary within which are fibers radiating perpendicularly from the cell wall (cw).

The cell wall after permanganate fixation shows the tripartite structure of the outer L IV layer and the plasma membrane (Fig. 26c). However, in contrast to A. nidulans (cf. Fig. 26a), there is an electron dense layer immediately inside layer L IV. This is taken to represent layer L III because of its position next to L IV.

Also to be found in Fig. 26c (arrows) is the elaboration of the thylakoidal membranes (t) from the plasma membrane (pm).

DISCUSSION

The results presented in this study generally support the contention of Moore and Dorward (1968), and others (Pearce, 1958, 1960; Tatum and Blackburn, 1962), that algae are capable of adapting to pesticides (Figs. 1 and 2). This is in contrast to a number of studies (Anonymous, 1963; Wurster, 1968; Menzel, Anderson and Randtke, 1970; Glooschenko, 1971; Derby and Ruber, 1971) employing short term techniques. These authors found a marked suppression of photosynthesis in susceptible species. Conclusions drawn from Table 1 lend support to these observations. However, Figs. 2, 3, 9 and 10 demonstrate that after 5 days rates of photosynthesis may approximate the control suggesting adaptive responses. Similar observations were made by Stadnyk, Campbell and Johnson, (1971) who suggested that emphasis in future studies be put on long term chronic effects.

In continuous culture, growth of Chlorella sp. was severely inhibited by DDT (Sodergren, 1968). However, this system maintains balanced growth so that the cell concentration remains constant. Thus the introduction of pesticide is maintained in constant ratio with the cells. This is not the case in the present study, and therefore no valid comparison can be drawn. Instead a closed system has been employed to simulate algal bloom conditions such as occur in Lake Erie. Furthermore, the validity of employing high concentrations of pesticide (6.5μg/ml) may be open to question. However, as pointed out previously (cf. Fig. 2, Wurster, 1968), the response of algae to pesticides is dose dependent. That is to say, the effect is not so much dependent on the absolute concentration of the pesticide as it is on the amount available per cell. Thus the observations reported herein could probably have been obtained employing 1 ng/ml pesticide and an initial concentration of 10^2 cells/ml of M. aeruginosa instead of 6.3×10^5 cells/ml. The former conditions represent approximately

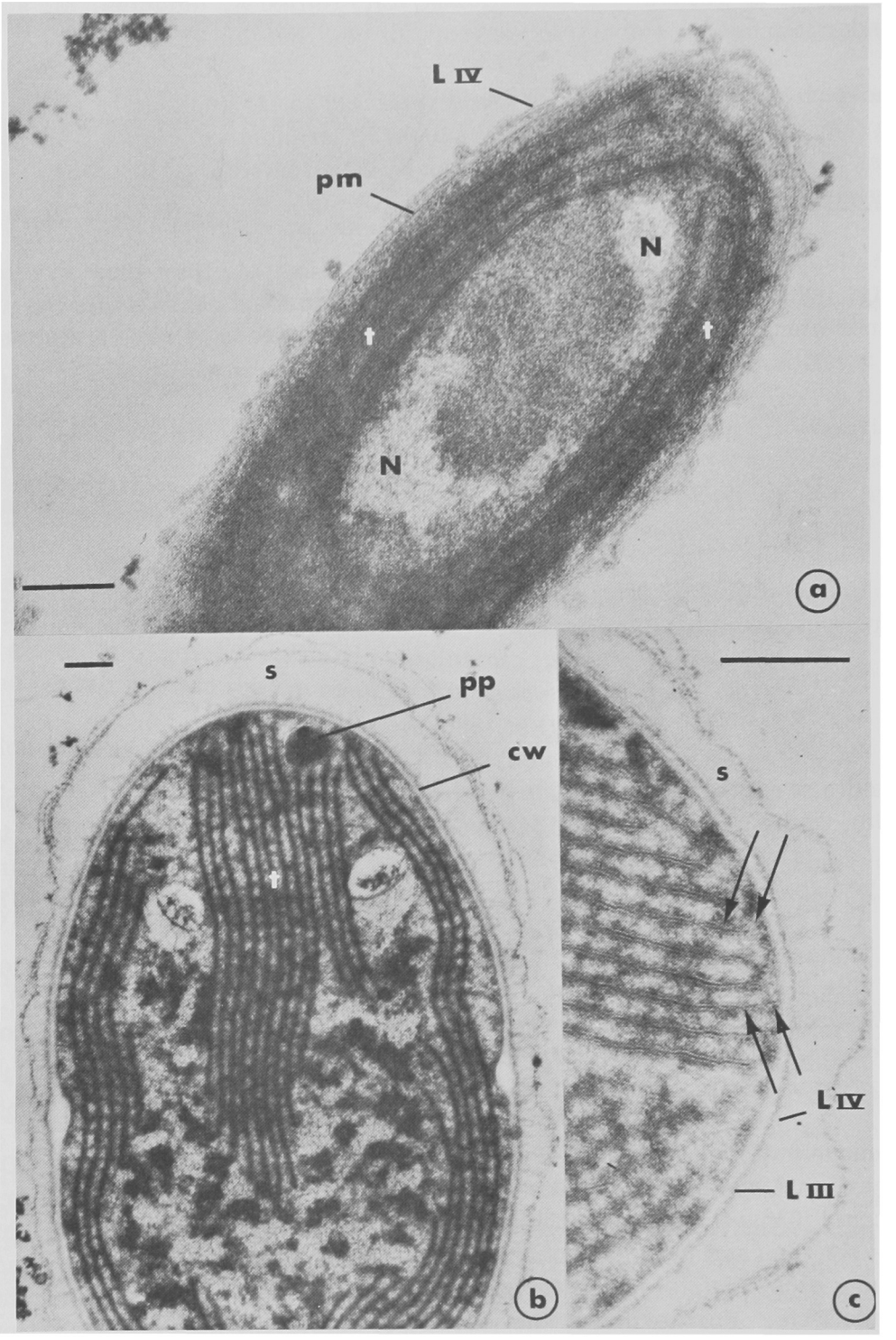

Fig. 26 -- Permanganate fixation of A. nidulans, a. The L IV layer, cell wall (cw), plasma membrane (pm), M. aeuginosa fixed with permanganate showing the sheath (s), cell wall (cw), thylakoids (t) and polyphosphate granule (pp). c, enlarged micrograph of M. aeruginosa showing the origin of the thylakoids (t) from the plasma membrane (arrows) and also the L III and I IV layers of the cell wall. Embedded in Epon. Bar equals 0.2 μm.

2 orders of magnitude increase over the aldrin associated with Lake Erie microparticulates (Pfister, Frea and Dugan, 1969) and the approximate concentration of M. aeruginosa just prior to an algal bloom (John Gnau, personal communication). The 6.5 μg/ml pesticide added initially in this study was equivalent to 10^{-11}g/cell for M. aeruginosa and 1.12 x 10^{-12}g/cell for A. nidulans, a close approximation to the natural situation. (A note of clarification: There is a point below which the dilution of both the pesticide and alga will be so great that they will not interact. Furthermore, for daily addition studies the inoculum was increased to facilitate measurement of oxygen evolution. This resulted in a decreased pesticide to cell ratio.)

Daily addition of pesticides has provided insight into the mechanisms of their action on A. nidulans. Both aldrin and dieldrin suppressed the rate of oxygen evolved per cell (Fig. 6), but the production of oxygen per unit chl a of aldrin-treated cells was almost equal to the control (Fig. 7). Aldrin and dieldrin inhibited the synthesis of chl a of the cells (Fig. 8). This inhibition was reversed in dieldrin-treated cells toward the end of the test period while the rate of oxygen evolution decreased (Fig. 7).

When aldrin affects photosynthesis, it appears to be through the inhibition of chl a synthesis. Initially dieldrin acted in a similar manner, ultimately inhibiting the photosynthetic process per se. It is known that some pesticides interfere with ATPase activity (Matsumura and Patil, 1969). Since ATP formation is coupled to photosynthesis (Jagendorf and Uribe, 1967), dieldrin may act as an energy transfer inhibitor. Such an interference mechanism would reduce the rate of oxygen evolution; this has been observed here with dieldrin. What ever the mechanism the trend for cultures treated with pesticide was one of adaptation and recovery.

That the effect on growth was delayed is demonstrated in Fig. 5. By the time dieldrin inhibited growth only 3.3 generations had occurred. It is, therefore, not inconceivable for the effect on growth to appear negligible. While aldrin suppressed chl a synthesis, the possibility exists that the reduction was not sufficient to alter growth from the control.

When pesticides were added daily to cultures of M. aeruginosa, the cell numbers were immediately affected (Fig. 13). Although aldrin and dieldrin stimulated photosynthesis on a per cell basis (Fig. 14), the rate of oxygen evolved per unit chl a was reduced. As with A. nidulans, the trend was toward adaptation and recovery.

Previous work on bioconcentration of pesticides by blue-green algae reported concentration factors after varying periods of exposure (Gregory, Reed and Priester, 1969; Vance and Drummond, 1969). The present study investigated the fate of the pesticides over a 20-day period. Both A. nidulans and M. aeruginosa accumulated dieldrin on the first day (Figs. 18 and 20). During the early stages of growth, the pesticide was released to the surfaces of the cells. Dieldrin was then reabsorbed by cells of A. nidulans. Reabsorption did not occur in cultures of M. aeruginosa. Possibly bacteria in the culture metabolized dieldrin while it was on the surface of the cells.

We propose that cells from the mid and late log periods of growth are capable of actively excluding pesticide. Growth during these periods can be regarded as a reflection of high

metabolic rates. The possibility exists that production of exopolymer, which comprises the sheath, during these periods of high metabolic activity may act as a carrier to extrude the absorbed pesticide. If, as Sodergren (1968) suggests, the mechanism of uptake is passive absorption, cells of lower metabolic activity from the lag and stationary phases of growth would show a marked increase in pesticide accumulation. A similar situation probably exists for aldrin (Figs. 17 and 19).

The ultrastructure of neither M. aeruginosa nor A. nidulans was apparently altered by the addition of pesticides. A change in the thylakoids would have supported the hypothesis that aldrin inhibits the synthesis of chl a. However, the slight differences in growth in response to the pesticide predicted our cytological observations. Had growth been reduced by an order of magnitude identification of structural changes would have been facilitated. However, in lieu of defining any difference in structure as a result of pesticide treatment, a number of cytological observations have been made.

The layers of the cell wall described by Jost (1965) are apparent in thin sections of A. nidulans (cf. Allen, 1968b) and M. aeruginosa. Allen (1968b) described an irregular thin electron dense layer, apposed to L IV of the cell wall, which was suggested to be a sheath. This layer was not common to all thin sections she presented. The irregular and inconsistent appearance of the sheath suggests the possibility of the adsorption of some contaminant. None of the electron micrographs in this study suggest the existence of a sheath in A. nidulans (Figs. 21-23).

M. aeruginosa, in laboratory culture, possesses a small sheath which appears as a fibrous matrix with glutaraldehyde-osmium fixation (Figs. 24-25b, c). With permanganate fixation (Figs. 26b, c) and freeze-etching replicas (Johnson, 1970) the sheath appears to possess fibrils radiating from the cell wall. In the natural environment, the sloughed off sheath may serve as a carbon source for bacteria (Tuffery, 1969; Johnson, 1970).

The profile of the cell wall in glutaraldehyde-osmium fixation shows layers L I, L II, L III, and L IV in A. nidulans as well as M. aeruginosa. The L I layer is somewhat irregular in thickness in M. aeruginosa (Fig. 25b) and may be absent in A. nidulans (Fig. 23a). These findings corroborate the conclusion drawn by Allen (1968b) that L I is an artifact resulting from electron microscopic preparation.

A comparison of the cell wall of both blue-green algae fixed with permanganate shows different electron images. Inside L IV of M. aeruginosa is an electron dense layer which is not observable in A. nidulans. The location in the wall indicates that it may be the L III layer and that this layer is of different composition than its counterpart in A. nidulans. Since M. aeruginosa possesses a sheath, L III may be the site of sheath synthesis.

Strong evidence is provided in Fig. 26c to indicate a connection between the thylakoids and the plasma membrane of M. aeruginosa. This association suggests that the photosynthetic apparatus arises as the result of an elaboration of the plasma membrane.

Cell division takes place via an invagination of the plasma membrane which is followed by a centripedal proliferation of L II (Allen, 1968b). The mode of division is similar for

both A. nidulans and M. aeruginosa although the latter undergoes more of a constrictive process (Figs. 23a and 24a).

The thylakoids in M. aeruginosa occasionally appear as layered stacks (Fig. 26b). The division plane has always been observed to be perpendicular to the thylakoids and the longitudinal axis. This suggests that division occurs in one plane.

We have observed spherical masses of membranes (Fig. 23c) wound in concentric circles which were called "lamellosomes" by Echlin (1964b) to signify their mesosomal structure. That these are not reducing sites has recently been demonstrated (Bisalputra, Brown and Weier, 1969). Because of their similar structure and staining properties they have been included as cyanophycin granules by Lang (1968). In addition to observing them free in the cytoplasm they have also been found in association with polyphosphate granules (Fig. 23a). The deposition of polyphosphate within the cyanophycin granule (Fig. 23c) is strongly suggestive of a functional relationship between the two structures. Thus it seems feasible that the cyanophycin granule may serve as a site for the formation of polymeric phosphate.

SUMMARY

1. Growth of A. nidulans was initially inhibited by both aldrin and dieldrin. On continued incubation A. nidulans was able to recover from both treatments.

2. Production of oxygen was inhibited only by dieldrin. This effect was not immediate but occurred after 24 hr incubation.

3. Aldrin and dieldrin appear to exhibit different mechanisms of inhibition of A. nidulans. Aldrin inhibits the amount of chlorophyll synthesized; dieldrin suppresses the photosynthetic apparatus.

4. Cell numbers were reduced by a single or daily addition of dieldrin to cultures of M. aeruginosa.

5. The general trend in response to pesticide treatments was toward adaptation and recovery.

6. Pesticides were concentrated by both A. nidulans and M. aeruginosa. Aldrin and dieldrin were accumulated initially by both organisms but were released and later reabsorbed.

7. Attempts to locate ultrastructurally the site(s) of inhibition by the pesticides have been unsuccessful.

8. There has been a variation in the fixation image of A. nidulans. The cause is not known at this time.

9. The L I, L II, L III, L IV layers of the cell wall of blue-green algae have been shown for the organisms studied.

10. Cell division occurs via centripedal proliferation of L II.

11. Cyanophycin granules may be the site of polymeric phosphate synthesis.

12. The thylakoids in M. aeruginosa appear to arise from the plasma membrane.

13. The L IV appears similar to the plasma membrane when $KMnO_4$ is used as a fixative.

LITERATURE CITED

Acree, F., Jr. 1963. Insecticide volatility, codistillation of DDT with water. J. Agr. Food Chem. 11: 278.

Alexander, M. 1967. The breakdown of pesticides in soils, p. 331-342. In N.C. Brad (ed.), Agriculture and the quality of our environment. Am. Assoc. Ad Sci., Washington, D.C.

Allen, M.M. 1968a. Photosynthetic membrane system in Anacystis nidulans. J. Bacte 96: 831-836.

Allen, M.M. 1968b. Ultrastructure of the cell wall and cell division of unicellular blue green algae. J. Bacteriol. 96: 842-852.

Allen, M.M. 1968c. Simple conditions for growth of unicellular blue-green algae on pl J. Phycol. 4: 1-4.

Allen, M.M. and R.Y. Stanier. 1968a. Growth and division of some unicellular blue-g algae. J. Gen. Microbiol. 51: 199-202.

Allen, M.M. and R.Y. Stanier. 1968b. Selective isolation of blue-green algae from wa and soil. J. Gen. Microbiol. 51: 203-209.

Anonymous, 1963. Pesticide-wildlife studies. U.S. Department of the Interior, Fish a Wildlife Service, Circ. 167.

Bisalputra, T., D.L. Brown and T.E. Weier. 1969. Possible respiratory sites in a blue-green alga Nostoc sphaericum as demonstrated by potassium tellu and tetranitroblue tetrazolium reduction.

Bollen, W.B. 1961. Interactions between pesticides and soil microorganisms. Ann. R Microbiol. 15: 69-72.

Chacko, C.I. and J.L. Lockwood. 1967. Accumulation of DDT and dieldrin by micro-organisms. Can. J. Microbiol. 13: 1123-1126.

Cohen-Bazire, G., R. Kunisawa and N. Pfennig. 1969. Comparative study of the struc of gas vacuoles. J. Bacteriol. 100: 1049-1061.

Cox, J.L. 1970a. DDT residues in marine phytoplankton: Increase from 1955-1969. Science 170: 71-73.

Cox, J.L. 1970b. Low ambient level uptake of ^{14}C-DDT by three species of marine ph plankton. Bull. Environ. Contamination Toxicol. 5: 218-221.

Derby, S. B. and E. Ruber. 1971. Primary production: Depression of oxygen evolution in algal cultures by organophosphorous insecticides. Bull. Environ. Contamination Toxicol. 5: 553-558.

Drews, G. and H. Meyer. 1964. Untersuchungen zum chemischen Aufbau der Zellwände von Anacystis nidulans und Chlorogloea fritschi. Arch. Mikrobiol. 48: 259-267.

Drews, G. and W. Niklowitz. 1956. Beiträge zur Cytologie der Blaualgen. III. Untersuchungen uber die granularen Einschlusse der Hormogonales. Arch. Mikrobiol. 25: 333-351.

Drews, G., H. Prauser and D. Ulmann. 1961. Massenvorkomenn von Synechoccus placticus nov. spec., einer solitaren, planktischen Cyanophycee, in einem Abwasserteich. Arch. Mikrobiol. 39: 101-115.

Echlin, P. 1964a. The fine structure of the blue-green alga Anacystis montana f. minor grown in continuous illumination. Protoplasma 58: 439-457.

Echlin, P. 1964b. Intracytoplasmic membranous inclusions in the blue-green alga, Anacystis nidulans. Arch. Mikrobiol. 49: 267-274.

Edwards, M.R., D.S. Berns, W.C. Ghiorse and S.C. Holt. 1968. Ultrastructure of the thermophylic blue-green alga, Synechoccus lividus Copeland. J. Phycol. 4: 283-298.

Frank, V.H., M. Lefort and H.H. Martin. 1962. Elektronenoptische und chemische Untersuchungen an Zellwänden der Blaualga Phormidium uncinatum. Z. Naturforsch. 176: 262-268.

Freeman, J.A. and B.O. Spurlock. 1962. A new epoxy embedment for electron microscopy. J. Cell. Biol. 13: 437-443.

Gantt, E. and S.F. Conti. 1967. Phycobiliprotein localization in algae. Brookhaven Symp. Biol. 19: 393-405.

Gantt, E. and S.F. Conti. 1969. Ultrastructure of blue-green algae. J. Bacteriol. 97: 1486-1493.

Giesy, R.M. 1964. A light and electron microscope study of interlamellar polyglucoside bodies in Oscillatoria chalybia Am. J. Bot. 51: 388-396.

Glooschenko, W.A. 1971. The effect of DDT and dieldrin upon ^{14}C uptake by in situ phytoplankton in lakes Erie and Ontario. 14th Great Lakes Conference.

Gregory, W.W., Jr., J.K. Reed and L.E. Priester, Jr. 1969. Accumulation of parathion and DDT by some algae and protozoa. J. Protozool. 16: 69-71.

Hall, W.T. 1967. Ultrastructural studies on organelles of Glancocystis nostochinearum itzigsohn. J. Phycol. 3: 37-51.

Hill, D.W. and P.L. McCarty. 1967. Anaerobic degradation of selected chlorinated hydrocarbon pesticides. J. Water Polu. Con. Fed. 39: 1259-1277.

Holden, M. 1965. Chlorophylls. p. 466. In T.W. Goodwin (ed.), Chemistry and biochemistry of plant pigments. Academic Press, New York.

Jagendorf, A.T. and E. Uribe. 1967. Photophosphorylation and the chemiosmotic hypothesis. Brookhaven Symp. Biol. 19: 215-245.

Jensen, T.E. 1969. Fine structure of developing polyphosphate bodies in a blue-green alga, Plectonema boryanum. Arch. Mikrobiol. 67: 328-338.

Jensen, T.E. and C.C. Bowen. 1970. Cytology of blue-green algae II. Unusual inclusions in the cytoplasm. Cytologia 35: 132-152.

Johnson, A.J. 1970. A study of Microcystis aeruginosa and its relationship with associated bacteria. The Ohio State University, M.S. Thesis. 62 p.

Jones, D.D. and M. Jost. 1970. Isolation and chemical characterization of gas vacuole membranes from Microcystis aeruginosa Kentz. emend. Elenkin. Arch. Mikrobiol. 70: 43-64.

Jost, M. 1965. Die Ultrastructur von Oscillatoria rubescens D.C. Arch Mikrobiol. 50: 211-245.

Jost, M. and D.D. Jones. 1970. Morphological parameters and macromolecular organization of gas vacuole membranes of Microcystis aeruginosa Kuetz. emend. Elenkin. Can. J. Microbiol. 16: 159-164.

Kearney, P., R. Nash and A.R. Isensee. 1969. Persistence of pesticide residues in soils. p. 54-67. In. N. Miller and G. Berg (ed.), Chemical fallout. C.C. Thomas, Springfield, Ill.

Keil, J.E. and L.E. Priester. 1969. DDT uptake and metabolism by a marine diatom. Bull. Environ. Contamination Toxicol. 4: 169-173.

Kellenberger, E., A. Ryter and J. Sechaud. 1958. Electron microscope studies of DNA-containing plasms. II. Vegetative and mature DNA as compared with bacterial nucleoids in differing physiological states. J. Biophys. Biochem. Cytol. 4: 671-676.

Ko, W.H. and J.L. Lockwood. 1968. Conversion of DDT to DDD in soil and the effect of these compounds on soil microorganisms. Can. J. Microbiol. 14: 1069-1073.

Kratz, W.A. and J. Meyers. 1955. Nutrition and growth of several blue-green algae. Am. J. Bot. 42: 282.

Lamont, H.C. 1969. Shear-oriented microfibrils in the mucilaginous investments of two motile Oscillatoriacean blue-green algae. J. Bacteriol. 97: 350-361.

Lang, N.J. 1968. The fine structure of blue-green algae. Ann.Rev. Microbiol. 22: 15-46.

Lang, N.J. and K.A. Fisher. 1969. Variation in the fixation image of "structured granules" in Anabaena. Arch. Mikrobiol. 67: 173-181.

Leshniowski, W.O., P.R.Dugan, R.M. Pfister, J.I. Frea and C.I.Randles. 1970. Aldrin: Removal from lake water by flocculent bacteria. Science 169: 993-995.

Luft, J.H. 1956. Permanganate--a new fixative for electron microscopy. J. Biophys. Biochem. Cytol. 2: 799-802.

Luft, J.H. 1961. Improvements in epoxy embedding methods. J. Biophys.Biochem. Cytol. 9: 409-414.

Matsumura, F. and K.C.Patil. 1969. Adenosine triphosphate sensitive to DDT synapses of rat brain. Science 166: 121-122.

Menzel, D.W., J. Anderson and A.Randtke. 1970. Marine phytoplankton vary in their response to chlorinated hydrocarbons. Science 167: 1724-1726.

Menzie, C.M. 1969. Metabolism of pesticides. U.S. Department of the Interior, Fish and Wildlife Service, Report No. 127. 487 p.

Mercer, E.H. and M.S.C. Birbeck. 1966. Electron microscopy: A handbook for biologists. Blackwell Scientific Pub., Oxford, England.

Moor, R.B. and D.A. Dorward. 1968. Accumulation and metabolism of pesticides by algae. J. Phycol. 4: 7s.

Niemeyer, R. and G. Richter. 1969. Schnellmarkierte Polyphosphate und Metaphosphate bei der Blaualge Anacystis nidulans. Arch. Mikrobiol. 69: 54-59.

Pankratz, H.S. and C.C. Bowen. 1963. Cytology of blue-green algae. I. The cells of Symploca muscorum. Am. J. Bot. 59: 387-399.

Patil, K.C., F. Matsumura and G. Boush. 1970. Degradation of endrin, aldrin and DDT by soil microorganisms. Appl. Microbiol. 19: 879-881.

Peat, A. and B.A. Whitton. 1967. Environmental effects on the structure of the blue-green alga, Chlorogloea fritschii. Arch. Mikrobiol. 57: 155-180.

Pfister, R. M., P. R. Dugan and J. I. Frea. 1969. Microparticulates: Isolation from water and identification of associated chlorinated pesticides. Science 166: 878-879.

Pfister, R. M., J. I. Frea, P. R. Dugan, C. I. Randles, K. Zaebst, J. Duchene, T. McNair and R. Kennedy. 1970. Chlorinated hydrocarbon, microparticulate effects on microorganisms isolated from Lake Erie. Proc. 13th Conf. Great Lakes Res. pp. 82-92.

Pierce, M. 1958. The effect of the weedicide Kuron upon the flora and fauna of two experimental areas of Long Pond, Dutchess County, N. Y., N. E. Weed Control Conf. Proc. 12: 338.

Pierce, M. 1960. Progress report of the effect of Kuron upon the biota of Long Pond, Dutchess County, N. Y. N. E. Weed Control Conf. Proc. 14: 472.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17: 208-212.

Ris, H. and R. N. Singh. 1961. Electron microscope studies on blue-green algae. J. Biophys. Biochem. Cytol. 9: 63-80.

Smith, R. V. and A. Peat. 1967a. Comparative structure of the gas-vacuoles of blue-green algae. Arch. Mikrobiol. 57: 111-122.

Smith, R. V. and A. Peat. 1967b. Growth and gas-vacuole development in vegetative cells of Anabaena flos-aquae. Arch. Mikrobiol. 58: 117-126.

Smith, R. V., A. Peat and C. J. Bailey. 1969. The isolation and characterization of gas cylinder membranes and of granules from Anabaena flos-aquae. Arch. Mikrobiol. 65: 87-97.

Södergren, A. 1968. Uptake and accumulation of ^{14}C-DDT by Chlorella sp. (Chlorophyceae). Oikos 19: 126-138.

Stadnyk, L., R. S. Campbell and B. T. Johnson. 1971. Pesticide effect on growth and ^{14}C assimilation in a freshwater alga. Bull. Environ. Contam. Toxicol. 6: 1-8.

Starr, R. C. 1964. The culture collection of algae at Indiana University. Am. J. Bot. 51: 1013-1044.

Sweeney, R. A. 1965. Metabolism of lindane by Chlorella vulgaris and Chlamydomonas reinhardtii. J. Phycol. 4: 7s.

Tatum, W. M. and R. D. Blackburn. 1962. Preliminary study of the effects of diquat on the natural bottom fauna and plankton in two subtropical ponds. S. E. Assoc. Game and Fish Comm. Proc. Ann. Conf. p. 16.

Tuffery, A.A. 1969. Light and electron microscopy of a sheath of a blue-green alga. J. Gen. Mikrobiol. 57: 41-50.

Ukeles, R. 1962. Growth of pure cultures of marine phytoplankton in the presence of toxicants. Appl. Microbiol. 10: 532-537.

Van Baalen, C. and R.M. Brown, Jr. 1969. The ultrastructure of the marine blue-green alga, Trichodesmium erythaeum, with special reference to cell wall, gas vacuoles, and cylindrical bodies. Arch. Mikrobiol. 69: 71-91.

Vance, B.D. and W. Drummond. 1969. Biological concentration of pesticides by algae. J. Am. Water Works Assoc. 61: 360-362.

Van Meddelem, C.H. 1966. Fate and persistence of organic pesticides in the environment. p. 228-249. In A.A. Rosen and H.F. Kraybill (ed.), Organic pesticides in the environment. American Chemical Society, Washington, D.C.

Waaland, J.R. and Branton. 1969. Gas vacuole development in a glue-green alga. Science 163: 1339-1341.

Walsby, A.E. and B. Buckland. 1969. Isolation and purification of intact gas vesicles from a blue-green alga. Nature 224: 716-717.

Ware, G.W. and C.C. Roan. 1970. Interaction of pesticides with aquatic microorganisms and plankton. Residue Rev. 33: 15-45.

Wheeler, W.B. 1970. Experimental absorption of dieldrin by Chlorella. J. Agr. Food Chem. 18: 416-419.

Wurster, C.F. 1968. DDT reduces photosynthesis by marine phytoplankton. Science 159: 1474-1475.

Wurster, C.F. 1969. Chlorinated hydrocarbon insecticides and avian reproduction: How are they related? p. 368-387. In M. Miller and G. Berg (ed.), Chemical Fallout. C.C. Thomas, Springfield, Ill.